Use Of Adeno-associated Virus To Investigate Transcriptional Regulation Of The Preprotachykinin-A Promoter in Cultured DRG Neurons

by

Lesley Gerrard

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Declaration

I declare that this thesis has been composed by myself and that the studies presented are the results of my own independent investigations unless otherwise stated.
The work has not been and is not currently being submitted for candidature in any other degree or professional qualification.

Lesley Gerrard (Candidate)

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Abstract

The preprotachykinin-A (PPT-A) gene encodes the neuropeptides substance P, neurokinin A, neuropeptide K and neuropeptide γ, each derived by alternative mRNA splicing and post-translational processing of precursor polypeptides. The PPT-A gene is regulated by a variety of extracellular stimuli including NGF, GDNF, steroids, and inflammation and it is likely that many of these stimuli act at the level of transcription.

This study set out to investigate transcriptional regulation of the rat PPT-A gene promoter in dorsal root ganglia neurons (DRG). The proximal promoter region of the PPT-A gene has been extensively studied however, functional studies have been greatly hindered due to the lack of clonal cell lines, which express endogenous PPT-A or can support PPT-A promoter function in reporter gene constructs. DRG express the PPT-A gene endogenously however neurons are refractory to all methods of transfection. Adeno-associated virus vectors (AAV) were therefore used as a tool for the transduction of PPT-A promoter fragments driving a reporter gene to investigate PPT-A promoter activity.

AAV vectors were shown to achieve 100% transduction efficiency of both neuronal and nonneuronal cells in DRG cultures and AAV vectors containing the PPT-A promoter demonstrated a more restricted expression pattern. In addition, AAV vectors allowed long-term reporter gene expression in cultured DRG neurons in which expression was predominately neuronal.

The definition of functional promoter elements supporting tissue specific expression of the PPT-A gene was firstly analysed by infection of adult and neonate cultured DRG neurons with AAV vectors that contained different lengths of the PPT-

A proximal promoter driving the luciferase reporter gene. The PPT-A promoter was found to contain elements that are differentially regulated between neonate and adult DRG neurons. Secondly, the effect of growth factors NGF, LIF, IL-6, BDNF and GDNF on the PPT-A promoter fragment (nucleotides –865 to +92) were examined in cultured adult DRG neurons. These growth factors are known to alter neuropeptide levels and neuronal phenotype. It was found that NGF, LIF and GDNF independently acted to influence PPT-A promoter activity in adult DRG neurons. This suggests that the proximal PPT-A promoter contains regulatory elements that can mediate the effects of these growth factors.

Furthermore, the role of a specific binding motif for the basic helix-loop-helix (bHLH) family of transcription factors was addressed. Previous biochemical and cell line models have implicated this motif as an important regulator of PPT-A promoter activity. The activity of the PPT-A promoter fragment –865+92 containing an insertional mutation at nucleotide –60 disrupting the bHLH binding motif was compared to the wild-type promoter fragment in the presence or absence of NGF. It was established that this bHLH binding motif could be bound by a repressor in the absence of NGF and might be an important regulator of basal levels of PPT-A promoter activity. This suggests a novel mechanism by which bHLH factors might be important for NGF regulation of the PPT-A promoter.

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Abbreviations

A	adenine
AAV	adeno-associated virus
ABC	avidin:biotinylated complex
Ad	adenovirus
AP	alkaline phosphatase
AP1	activator protein 1
BDNF	brain derived neurotrophic factor
bHLH	basic helix-loop-helix
bp	base pair(s)
BSA	bovine serum albumin
bZIP	basic leucine zipper
C	cytosine
Ci	curies
CGRP	calcitonin gene related peptide
CMV	cytomegalovirus
CNS	central nervous system
CNTF	ciliary derived neurotrophic factor
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding proteins
DAG	diacylglycerol
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dH_20	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DRG	dorsal root ganglia
dT	2'-deoxythymidine
dTTP	2'-deoxythmidine-5'-triphosphate
EDTA	sodium ethylenediamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
FCS	foetal calf serum
g	gram(s)
G	guanine
GDNF	glial cell derived neurotrophic factor
GF	growth factor
GFP	green fluorescent protein
gp	glycoprotein
h	hour(s)
HEK	human embryonic kindey
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSH	helix-span-helix
HSV-1	herpes simplex virus-1
IB-4	isolectin B-4
IgG	immunoglobulin G

IL-6	interleukin-6
ip	infectious particle(s)
IP3	inositol 1,4,5-triphosphate
ITR	inverted terminal repeat
Jak	Janus kinases
kb	kilobase pairs
l	litre(s)
LB	L-broth
LIF	leukemia inhibitory factor
LUC	luciferase
M	molar
mg	milligram(s)
MHV	mouse herpes virus
min	minutes
ml	millilitre(s)
mm	millimetre
mM	millimolar
moi.	multiplicity of infection
mRNA	messenger ribonucleotide
mV	millivolt(s)
N	unspecified nucleotide
n	nano
NF	neurofilament
NF-κb	nuclear factor kappa b
NGF	nerve growth factor
NKA	neurokinin A
NKB	neurokinin B
NSCL	neurological stem cell leukemia factor
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
Npγ	neuropeptide γ
NpK	neuropeptide K
NPY	neuropeptide Y
ORF	open reading frame
^{32}P	phosphorus 32 radioisotope
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenamine
pfu	plaque forming units
PLC	phosphlipase C
PNS	peripheral nervous system
POU	pituitaryoctamer and unc
PPT-A	preprotachykinin-A
rAAV	recombinant adeno-associated virus
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SP	substance P

STAT	signal transducer and activator of transcription
T	thymidine
TAFs	TBP-associated factors
TBP	TATA-binding protein
$TCID_{50}$	tissue culture 50% infectious dose
TF	transcription factor
TPA	phorbol 12-myristate
Tris	tris(hydroxymethyl)aminoethane
Trk	tyrosine kinase
USF	upstream stimulatory factor
UV	ultraviolet
wtAAV	wild-type adeno-associated virus
w/v	weight/volume

Greek symbols

α	alpha
β	beta
γ	gamma
δ	delta
μ	micro

CHAPTER 1: INTRODUCTION

1.1 Tachykinins

This study set out to investigate the transcriptional regulation of the rat preprotachykinin-A (PPT-A) gene in rat dorsal root ganglia (DRG). The PPT-A gene encodes the precursors of the tachykinins that are a family of structurally related neuropeptides. The tachykinins are widely but selectively distributed throughout the central and peripheral nervous system.

The first tachykinin member to be discovered was substance P in 1931 by Von Euler and Gaddum and its structure was later elucidated by Chang *et al.* (1971). Until 1983, substance P was the only representative of the mammalian tachykinins, consequently it has been the most extensively characterised. Several other tachykinins have now been identified including neurokinin A (Nawa *et al.*, 1984; Krause *et al.*, 1987), neurokinin A (3-10) (Tatemoto *et al.*, 1985), neurokinin B (Kotatini *et al.*, 1986), neuropeptide γ (Kage *et al.*, 1988) and neuropeptide K (Tatemoto *et al.*, 1985).

1.1.1 Genes encoding tachykinins

Substance P (SP), neurokinin A (NKA), neurokinin A (3-10) (NKA (3-10)), neuropeptide K (NPK) and neuropeptide γ (NPγ) are encoded by the preprotachykinin-A gene (PPT-A). Neurokinin B (NKB) is encoded by the preprotachykinin B gene. The rat PPT-A gene is approximately 8 kb in length, containing 7 exons and 6 introns (Nawa *et al.*, 1984).

The primary RNA transcript produced from the PPT-A gene is alternatively spliced to produce 4 mRNAs (figure 1.1): α-, β-, γ- and δ-PPT mRNA, with each mRNA differing in exon usage. βPPT mRNA contains all seven of the PPT-A gene exons. Exon 6, which encodes NKA, is absent in α-PPT mRNA, whereas exon 4 is absent in γ-PPT mRNA (MacDonald *et al.*, 1989; Carter and Krause, 1990). Another splicing variant of the rat PPT-A gene is δ-PPT mRNA, which lacks both exon 4, and exon 6 (Harmar *et al.*, 1990). The precursor polypeptides produced from these mRNAs undergo post-translational modification to produce the corresponding neuropeptides. Part of exon 3 encodes substance P and therefore all mRNAs can produce SP, whereas NKA will be produced only from β- and γ-PPT precursors. NPK and NPγ are N-terminally extended derivatives of NKA and are the final products of post-translational modification of β- and γ-PPT in some tissues (Tatemoto *et al.*, 1985; Kage *et al.*, 1988).

Alternative RNA splicing is regulated in a tissue specific manner so the amounts of mRNA and therefore the amount of SP and NKA vary in different tissues. In the rat, α-PPT levels are relatively low in all central nervous system (CNS) and peripheral nervous system (PNS) tissues examined (<0.1%), whereas γ-PPT mRNAs are the most abundant (80%). β- and δ-PPT mRNAs have been shown to be present at similar levels (20%) in the PNS and CNS (Sivam *et al.*, 1989; Harmar *et al.*, 1990).

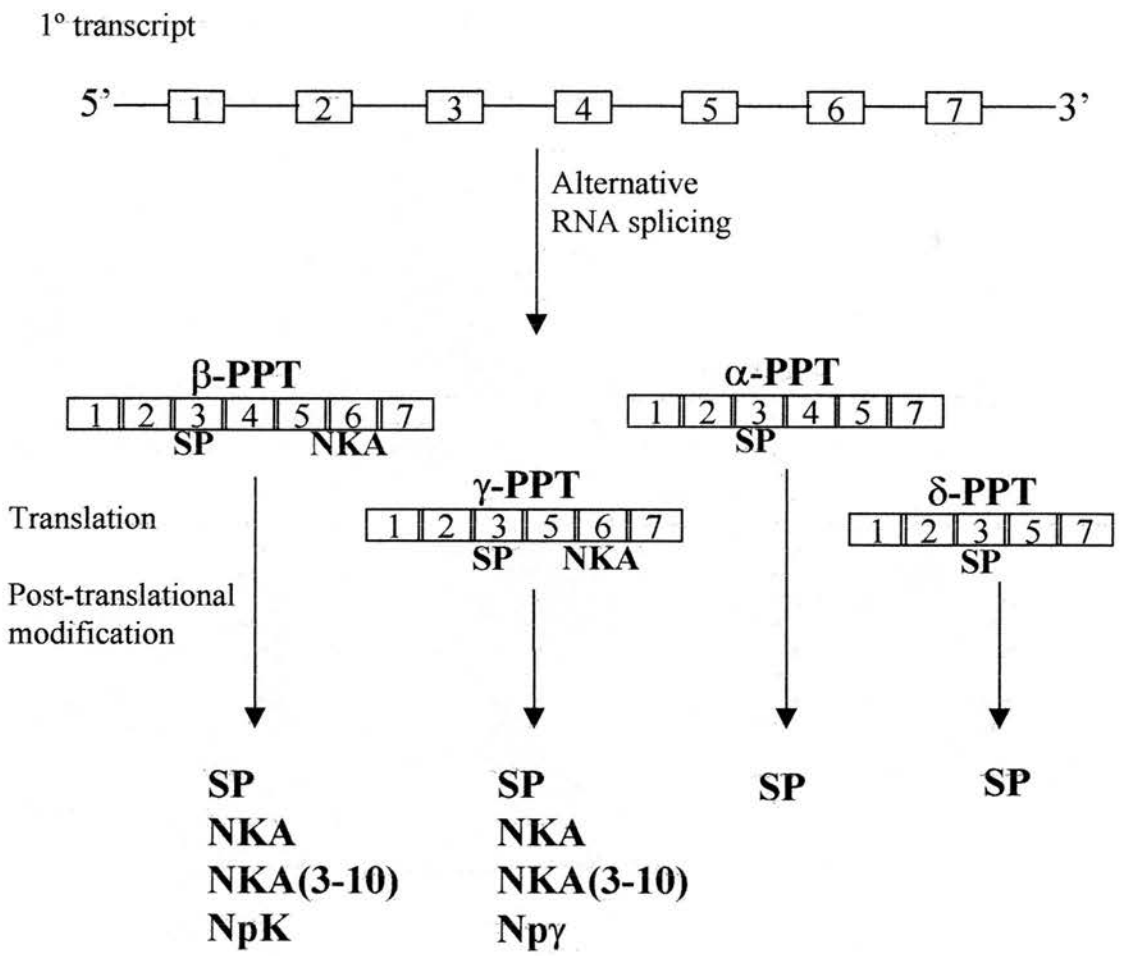

Figure 1.1: Schematic diagram of the PPT-A gene. Boxes and numbers represent the exons and horizontal lines represent the introns. Exon 3 contains the sequence information for SP and exon 6 contains the sequence information for NKA. Following alternative RNA splicing, 4 mRNA species are produced. The polypeptide precursors then undergo post-translational modification to generate neuropeptides SP, NKA, NKA(3-10), NpK and Npγ.

1.1.2 Distribution and role of tachykinins

Tachykinins are widely distributed throughout the mammalian body in both the central nervous system and peripheral tissues and numerous functions have been attributed to tachykinins in each of these tissues. SP and NKA are mostly synthesised in neurons of the peripheral and central nervous system where they are stored in large dense-core vesicles (McCarthy and Lawson, 1989). Upon excitation of these neurons, SP and NKA are released and act on their receptors on target cells to evoke various cellular responses. Consequently, a role for SP as a neurotransmitter was proposed (Otsuka and Yoshioka, 1993).

1.1.2.1 Central roles of tachykinins

Particular attention has been paid to the role of SP in the spinal cord, where it is highly concentrated in the neurons of the dorsal horn (McCarthy and Lawson, 1989). The dorsal horn neurons receive input from the DRG cells in the peripheral nervous system, which then process and relay somatosensory information to the CNS. PPT-A mRNA is expressed in approximately 20-30% of the dorsal root ganglia cells (Boehmer *et al.*, 1989). Most of these PPT-A containing neurons are small diameter cells (Tuchscherer and Seyboid, 1985) which give rise to the predominately nociceptive C- and Aδ-fibres. Subsequently, a role for SP as a neurotransmitter of afferent neurons in the spinal cord, and its involvement in pain transmission (nociception) has been the focus of many studies. Recently, mice with a disrupted PPT-A gene were studied (Cao *et al.*, 1998; Zimmer *et al.*, 1998). The mice generated by Cao *et al.* appeared to have normal thresholds for reactions to various painful stimuli, such as heat, mechanical pressure or chemical irritants. However,

there seemed to be a 'window' of painful intensities in which the animals showed reduced responses to the stimuli compared to the wild-type mice. The second study by Zimmer *et al.* (1998) demonstrated that the knock out animals show increased pain thresholds to certain painful stimulus, such as heat and noxious chemical stimuli, while other assays revealed no differences to the wild-type animal. Both studies demonstrate that tachykinins may play a role as a neurotransmitter in some but not all nociceptive responses, depending on the type and intensity of the painful stimuli. The difference in results obtained from these two studies could reflect the different genetic background of the mice studied or the experimental approaches taken. It has recently been shown that genetic variability of mice strains can affect the observed responses to common measures of nociception (Mogil *et al.*, 1999).

In addition to their roles in sensory transmission, tachykinins have been implicated in a variety of CNS functions such as the control of motor activities, autonomic and endocrine functions, and memory processings (Otsuka and Yoshioka, 1993). Recently, SP has also been implicated in the modulation of stress responses, mood, and anxiety (Rupniak and Kramer, 1999), but its exact role remains unclear. Localised administration of SP in the CNS may increase or reduce anxiety, depending on the animal species and location of injection (Teixeira *et al.*, 1996; File, 1997; de Araujo *et al.*, 1998). In humans, a SP receptor antagonist has been reported to be an effective antidepressant and anxiolytic (Kramer *et al.*, 1998). SP expression has also been studied in relation to several neurodegenerative disorders such as, Parkinson's disease (Gresch and Walker, 1999), Alzheimer's disease (Bouras *et al.*, 1990) and Huntington's disease (Richfield *et al.*, 1995) which are all associated with a progressive loss of SP expression within the brain.

1.1.2.2 Peripheral roles of the tachykinins

The majority of SP synthesised in neurons of the DRG is transported to the periphery (Harmar and Keen, 1984). SP has been found in a variety of peripheral nerves, including the phrenic (Malthe-Sorensen and Oktedadlon, 1982) and sciatic nerves (Lembreck *et al.*, 1981). Additionally, SP–containing nerves have been demonstrated in skin (Dalsgaard *et al.*, 1988), the respiratory, urinary and gastrointestinal tracts (Lecci *et al.*, 2000) and spleen, thymus and lymph nodes (Fink and Weihe, 1988; Popper *et al.*, 1988; Weihe *et al.*, 1989; Kurkowski *et al.*, 1990).

Peripheral SP is also thought to be involved in processes such as arthritis associated neurogenic inflammation (Donaldson *et al.*, 1992; Smith *et al.*, 1993), stimulation of immune function (Bar-Shavit *et al.*, 1980) and growth of fibroblasts and smooth muscle (Nilsson *et al.*, 1985). In addition, peripheral SP can function in hypertension, smooth muscle contraction and cellular proliferation (Lecci *et al.*, 2000). An increase in SP gene expression in DRG neurons has been associated with peripheral inflammation (Leslie *et al.*, 1995) and neuropathic pain (Noguchi *et al.*, 1994; Fukuoka *et al.*, 1998).

1.1.3 Regulation of the tachykinins

Tachykinins and their precursor mRNAs have been shown to display a significant degree of plasticity in expression levels. The term plasticity is used to describe short or long-lasting changes in neuronal phenotype in response to different stimuli. Much work has been carried out to define potential regulators involved in mediating plasticity of the PPT-A gene and its products in DRG neurons. A number of regulators have been determined and one such influencing factor is the

neurotrophin, nerve growth factor (NGF). NGF transported from the peripheral tissue regulates synthesis of SP in primary sensory neurons. NGF has been shown to regulate PPT-A gene expression in cultured DRG neurons (Lindsay and Harmar, 1989; Vedder *et al.*, 1993; Mulderry, 1994; Jiang and Smith; 1995) and *in vivo* (Leslie *et al.*, 1995; Otten and Lorez, 1983). These studies show that the addition of NGF can lead to an induction in SP expression or increased PPT-A mRNA production. In addition, the concentration of SP is decreased in sensory neurons following peripheral nerve transection or injury (Fitzgerald *et al.*, 1985; Noguchi *et al.*, 1989; Henken *et al.*, 1990; Noguchi *et al.*, 1993; Noguchi *et al.*, 1994; Noguchi *et al.*, 1995; Verge *et al.*, 1995). This suggests that SP synthesis is down-regulated by the depletion of NGF, which in the case of peripheral axotomy, is caused by the blockade of axonal flow in the peripheral branches of sensory neurons. Furthermore, peripheral inflammation is thought to influence SP levels in DRG neurons *in vivo* and is mediated by increased NGF expression (Cho *et al.*, 1996; Donaldson *et al.*, 1992; Donnerer *et al.*, 1992; Leslie *et al.*, 1995; Minami *et al.*, 1989; Woolf *et al.*, 1994). To assess whether the availability of endogenous NGF plays a role in triggering changes in PPT-A mRNA and SP levels, Shadiack *et al.* (2000) injected animals with antiserum against NGF. The results showed that SP levels were reduced in part suggesting NGF can influence these changes.

In addition to NGF, other neurotrophic factors can regulate SP expression in sensory neurons (Mulderry, 1994) and other stimuli have been shown to regulate PPT-A and SP levels in DRG neurons. These include capsaicin (Geppetti *et al.*, 1988; Jessell *et al.*, 1978; Kashiba *et al.*, 1997a) and long-term exposure to steroids (Liuzzi *et al.*, 1999) which act to decrease expression. In addition short term

exposure to steroids (Smith *et al.*, 1991) and morphine (Ma *et al.*, 2000) can increase SP expression.

1.1.4 Tachykinin receptors

The tachykinins mediate their effect by binding to G protein linked receptors that are characterised by the presence of a seven transmembrane domain. Upon binding the receptor, the tachykinins are believed to activate phospholipase C (PLC). This results in the stimulation of phosphatidyl inositol turnover and a resulting increase in intracellular inositol 1,4,5-triphosphate (IP3), which evokes Ca^{2+} release from intracellular stores. PLC activation can also result in protein kinase C (PKC) activation through diacylglycerol (DAG) (reviewed in Zeiglgansberger and Tolle, 1993). All of the tachykinins are able to induce similar biological actions, although their potency at inducing individual physiological responses differ suggesting they may act through different receptors. Ligand binding studies and molecular biology techniques have identified three tachykinin receptors NK1, NK2 and NK3, which are preferentially bound by SP, NKA and NKB respectively (Buck and Burcher, 1986; Masu *et al.*, 1987; Yokota *et al.*, 1989; Shigemoto *et al.*, 1990).

Recently mice which lack the gene encoding the NK1 receptor (De Felipe *et al.*, 1998; Laird *et al.*, 1999) or have had it specifically ablated with the toxin saporin (Mantyh, 1997) have shown that this receptor has an important role in hyperalgesia, pain responses and anxiety.

1.2 Transcriptional regulation of the PPT-A gene

1.2.1 Overview of transcription

The regulation of transcription is central both to tissue specific gene expression, and the regulation of gene activity in response to specific stimuli. Many of the factors acting to regulate PPT-A gene expression may be acting at the level of transcription. To understand the factors that are involved in mediating PPT-A mRNA synthesis, studies have focused on investigating transcriptional regulation of the promoter region of the PPT-A gene spanning nucleotides –3356 to +447. The initiation of transcription takes place through the interaction of defined proteins (transcription factors) with specific DNA sequences located on the promoter of a gene. The minimal promoter consists of basal promoter elements, which are the minimal DNA sequences necessary for transcription and can include a TATA box, or initiator elements. The TATA box is an AT-rich sequence, which is found approximately 30 bases upstream of the transcriptional start site in a variety of different genes (although is absent from some constitutively expressed and tissue-specific genes). It plays a critical role in positioning the start site of transcription. Initiator elements are found in the vicinity of the transcriptional start site of several eukaryotic genes and can serve a variety of functions and interact with a wide range of transcription factors (Du *et al.*, 1993; Seto *et al.*, 1991; Shi *et al.*, 1991). These basal elements interact with the basal transcription factors and this complex produces low rates of transcription. In addition, the promoter contains regulatory DNA elements, which bind other transcription factors to enhance or repress the rate of basal transcription. Eukaryotic promoters can span many kilobases, therefore these regulatory DNA elements may be located far from the basal promoter elements.

The PPT-A promoter spanning nucleotides –3356 to +447 has been previously characterised, as it is believed that the majority of important regulatory elements are located in this promoter fragment. It consists of basal promoter elements including a TATA box spanning nucleotides –22 to –28 (identified by Carter and Krause, 1990) and potential initiator elements located between nucleotides –20 to +4 (Mendelson, 1994). For transcription initiation, these basal promoter elements must bind the basal transcription machinery which comprises the general transcription factors TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF, TFIIH and TBP-associated factors (TAFs). These bind the DNA in a defined order to build a complex that is bound by RNA polymerase II (figure 1.2). The first step involves the binding of TBP and a variety of subunits termed TAFs, to a region that extends upstream of the TATA box. TFIIA also joins this complex and this is followed by TFIIB. TFIIF is next in order and this binds RNA polymerase II. Two final factors join the complex, TFIIE and TFIIH. This complex results in minimal transcription. However as stated earlier, the efficiency and specificity with which the promoter is recognised by transcription factors depends upon the regulatory DNA sequences. These bind regulatory transcription factors and ultimately result in the activation or repression of transcription (Latchman, 1995; Roeder, 1996) (figure 1.3).

In addition to the core promoter elements described above, this PPT-A fragment spanning nucleotides –3356 to +447 contains a number of potential regulatory DNA elements upstream and downstream of the transcriptional start site (Fiskerstrand and Quinn, 1996). These elements can bind transcription factors, which ultimately act to influence transcription positively or negatively. These DNA motifs may mediate the action of multiple stimuli. There are a limited number of such DNA

motifs that can occur in all promoters. However, the arrangements of different motifs within a promoter and the fact that different members of transcription factor families have the ability to recognise similar motifs can generate functional specificity. The various members can be regulated in a tissue-specific and tissue-inducible manner. The regulatory elements associated with the PPT-A promoter will be discussed below.

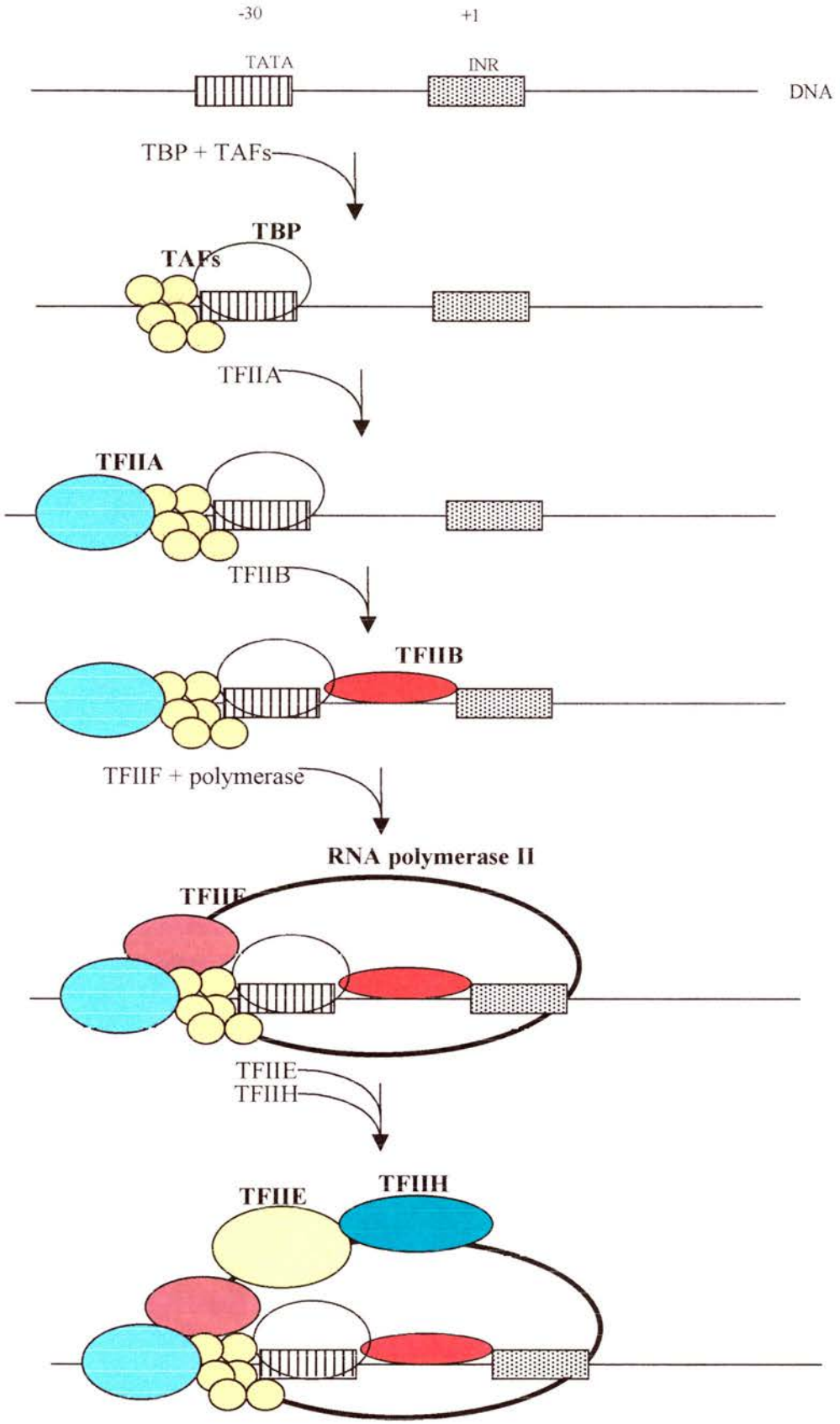

Figure 1.2: Assembly of the promoter initiation complex. The initiation complex assembles at promoters for RNA polymerase II by an ordered sequence of association with transcription factors. See text for details.

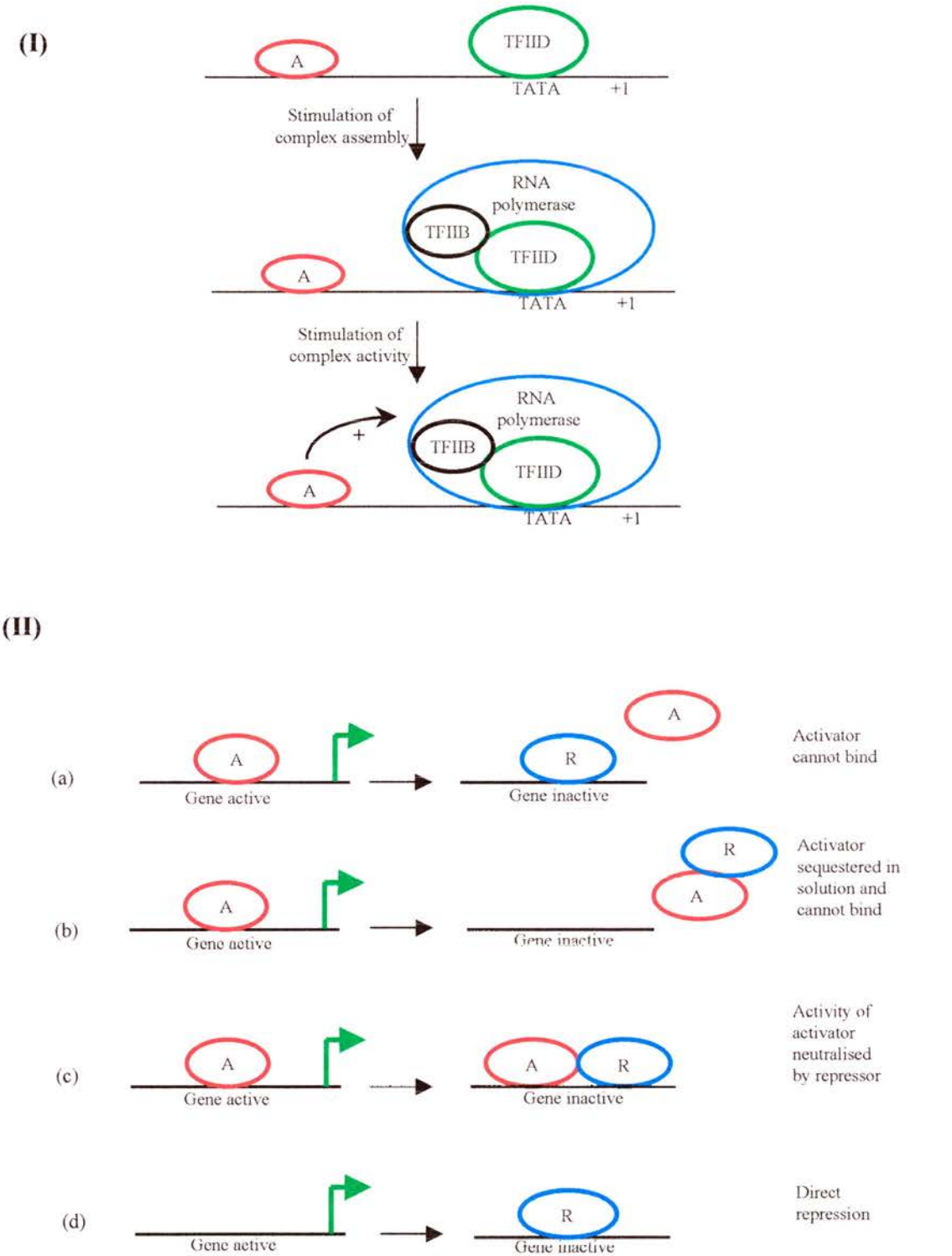

Figure 1.3: Mechanisms of (I) activation and (II) repression of gene expression. (I) An activator (A) bound to its binding site can stimulate either the assembly of the basal transcriptional complex consisting of RNA polymerase and its associated factors, or stimulate its activity once it has assembled. (II) Repression can occur by (a) the repressor (R) binding to DNA and preventing an activator from binding and activating gene expression; (b) by the repressor interacting with the activator in solution and preventing its DNA binding; (c) by the repressor binding to DNA with the activator and neutralising its ability to activate gene expression; or (d) by direct repression by an inhibitory transcription factor.

1.2.2 Potential transcription factors involved in PPT-A promoter regulation

Sequence analysis of the PPT promoter revealed several candidate DNA sequences that were related to known consensus sequences for binding transcription factors (Carter and Krause, 1990; Chapman *et al.*, 1993; Quinn, 1992 and Mendelson and Quinn, 1995). They include putative octamer binding protein sites, AP1 sites, cAMP response elements, GC boxes, and E box motifs. The transcription factor families, which are known to bind these consensus sequences, will first be described with specific reference to members that have been implicated in regulating PPT-A neuron specific gene expression. Subsequently, the experiments that have functionally defined the PPT-A promoter regulatory elements and implicated these transcription factors in regulating transcription of the PPT-A promoter will be discussed.

1.2.2.1 E box binding proteins

The E box binding proteins are a subset of a larger family of transcription factors termed the basic helix-loop-helix (bHLH) family and can bind the consensus sequence CANNTG. The actual sequence of a specific E box motif determines the affinity or specificity with which it will bind members of the E box binding proteins. The activities of these proteins are modulated by homodimer and heterodimer formation and they are often involved in tissue specific gene expression (Ball *et al.*, 1992; Murre *et al.*, 1989a; Therrien and Drouin, 1993). Neurons express specific bHLH factors in addition to a variety of more constitutively expressed bHLH factors such as upstream stimulatory factor (USF) and c-Myc and related proteins. Members that are specifically present in DRG neurons include neurological stem cell leukemia

factor (NSCL) (Begley *et al.*, 1992) and mammalian achaete-scute homologous-1 (MASH-1), the latter protein being upregulated in PC12 cells by NGF (Johnson *et al.*, 1990). Heterodimerization between ubiquitous and cell-lineage specific family members has been proposed as a mechanism for the regulation of tissue specific gene expression (Murre *et al.*, 1989b).

1.2.2.2 AP1 and CRE protein binding families

AP1 and cyclic AMP response element binding proteins (CREB) are subfamilies of the basic leucine zipper (bZIP) family of transcription factors. Commonly, members contain a 35 amino acid domain in which every seventh amino acid is a leucine. The collective term AP-1 is used to describe dimeric transcription factors composed of Jun and Fos subunits that bind to a common DNA site, the AP1 binding site ($TGA^{G}/_{C}TCA$). Members of the CREB family bind the cAMP reponse element (CRE) (TGACGTCA). All members of this bZIP family can form homo or heterodimers with members of their own subfamily or other families. Jun proteins form very stable heterodimers with Fos and CREB family members or can homodimerise among themselves (Kouzarides and Ziff, 1988; Smeal *et al.*, 1989; Hai and Curran, 1991). The resulting dimers are thought to possess different DNA binding specificities and affinities allowing targeting to different but related DNA sites. In both AP1 and CRE sites the structure which the consensus sequence and flanking DNA can adopt can play a role in determining both the identity of the specific complex that binds and the functional consequence of binding (Franza *et al.*, 1988).

NGF modulates CREB family members, which in turn regulates CRE activity (Ginty *et al.*, 1994; Hawley *et al.*, 1992). Jun has also been demonstrated to be regulated by NGF in DRG neurons (Gold *et al.*, 1993). Induction of Fos by NGF in PC12 cells is well established (Kruijer *et al.*, 1985; Curran and Franza; 1988; Curran and Morgan, 1985; Milbrant, 1986) and other growth factors have been shown to induce Fos in primary neuronal cultures of varying types (Collazo *et al.*, 1992; Engele and Schilling, 1996; Bilsland and Harper, 1998). In addition, compositional variations of proteins binding to AP1 sites changes in response to stimuli including NGF (Gizang-Ginsberg and Ziff, 1994; Quinn *et al.*, 1989) and can vary in a tissue specific manner (Andrews *et al.*, 1993).

1.2.2.3 Octamer binding proteins

Octamer binding proteins are part of a larger family of pituitary octamer and unc (POU) proteins, which contain both a homeobox-like sequence and a conserved POU domain (Herr *et al.*, 1988). This POU domain is important for site-specific DNA binding and protein-protein interactions between POU domain proteins and other transcription factors. Members of the octamer binding protein family recognise and bind to the octameric DNA consensus sequence ATTTGCAT. It has been shown that the consensus sequence is highly degenerate (Baumruker *et al.*, 1988) with flanking sequences also being important for mediating protein/DNA interactions.

Members serve a number of different functions and each show varying and overlapping tissue distributions (Herr *et al.*, 1988). POU domain proteins include the ubiquitously expressed Oct-1 protein and a number of nervous-tissue specific members including Brn-1, Brn-2, Brn-3a, 3b and Brn-4. These proteins can act as

both transcriptional repressors and activators (Morris *et al.*, 1997). Brn-3a and Brn-3b differ in their effect on a number of neuronal specific promoters (Morris *et al.*, 1997). Adult DRG neurons are known to express a number of POU-domain proteins including Oct-1, Oct-2 and Brn-3 and levels of Oct-2 are increased in DRG following treatment with NGF (Wood *et al.*, 1992; Kendell *et al.*, 1995).

1.2.2.4 Zinc finger transcription factors

Members of the zinc finger family of transcription factors contain a 30 amino acid zinc finger motif, which is present in variable numbers in many regulatory proteins (Rhodes and Klug, 1993). Members of the zinc finger family of transcription factors include Sp1, which can recognise and bind purine-rich and G-rich regions of DNA (Lania *et al.*, 1997). Sp1 binding sites are often found near binding sites for other transcription factors suggesting these factors act in conjunction to modulate transcription. Another member, Egr-1 has been shown to mediate growth factor and phorbol 12-myristate (TPA) activation of transcription (Christy *et al.*, 1988; Sukhatme *et al.*, 1988).

1.2.2.5 Helix-span-helix (HSH) transcription factors

Members of the HSH family of transcription factors contain a region rich in basic residues essential for DNA binding and dimerisation. Members include AP2 (Williams and Tjian, 1991) that recognizes sequences rich in dG nucleotides. AP2 has been detected in DRG neurons (Mitchell *et al.*, 1991) and expression in DRG neurons *in vivo* has been correlated with increased levels of PPT-A gene expression in an animal model of arthritis (Donaldson *et al.*, 1993).

1.2.3 Analysis of the PPT-A promoter

Investigating transcriptional regulation of the PPT-A promoter has been difficult, due to the lack of cell lines which endogenously express the gene or will support expression from transfected plasmids containing fragments of the PPT-A promoter driving reporter gene expression (Wood *et al.*, 1990; Mulderry *et al.*, 1993; Morrison *et al.*, 1994a). In addition, DRG primary cultures are generally resistant to transfection. Therefore, two strategies have been employed to investigate the transcriptional regulation and stimulus inducibility of the PPT-A promoter. Firstly, reporter gene constructs containing the PPT-A promoter were microinjected into cultured DRG neurons since DRG neurons express endogenous PPT-A and demonstrated activity of the PPT-A promoter (Mulderry *et al.*, 1993). It was found that the promoter fragment spanning base pairs –3356 to +447 was three times more active than a fragment spanning –3356 to +92. The activity of promoter fragment spanning –3356 to +92 was equal to that of fragment –865 to +92, therefore it was concluded that the majority of positive regulatory elements in the PPT-A promoter were located between base pairs –865+92. Subsequent analysis focused on DNA elements within this domain (Mulderry *et al.*, 1993). In addition, Mulderry *et al.* investigated whether the PPT-A promoter was NGF regulated in DRG cultures. Reporter gene constructs containing promoter fragments spanning base pairs –3356 to +92 and –3356 to +447, were microinjected into DRG neurons and maintained in the presence or absence of NGF. No difference in reporter gene activity was found between NGF treated and untreated cultures. It has recently been proposed that this may be a reflection on the process of microinjection of DNA into the DRG cultures (Quinn *et al.*, 2000). Quinn *et al.* (2000) suggested that this technique of

microinjection might not be suitable for investigating potential growth factor responses in cultured DRG neurons.

Secondly, the PC12 cell line was used as a model for the analysis of reporter gene constructs. The PC12 cell line was derived from rat adrenal chromaffin cell (Greene and Tischler, 1976) and has been extensively used as a model for neuronal gene expression. In the presence of NGF, PC12 cells will develop many characteristics indicative of a neuronal phenotype (Greene and Tischler, 1976). The PPT-A promoter is inactive in PC12 cells under basal conditions (Morrison *et al.*, 1994a) however, DNA elements contained within the promoter can interact in a sequence specific manner with transcription factors present in PC12 cells (Quinn, 1992). Therefore small fragments from the PPT-A promoter linked to a heterologous reporter construct were used. This allowed the ability of individual PPT-A DNA elements to stimulate transcription in PC12 cells to be determined. Many individual elements were found to be both active and NGF regulated in PC12 cells (section 1.2.3.2).

As DRG cultures do not allow sufficient quantities of nuclear extract for use in DNA binding studies, PC12 and HeLa cell lines were used for the characterisation of DNA binding motifs and the transcription factor families binding these motifs. These cell lines contain a variety of consitutively expressed transcription factors that can interact with the PPT-A promoter. DNase1 footprinting studies identified potential DNA-protein interactions on the PPT-A promoter. Electrophoretic mobility shift assays (EMSA) allowed the investigation of sequence specific binding factors. The PC12 cell line was therefore a useful model for the identification of potential transcription factors, which can interact with the PPT-A promoter, and can allow

signal transduction pathways to be postulated. The PC12 cell line can respond to a variety of stimuli, which induce the synthesis of various neuropeptides and neurotransmitters (Lewis *et al.*, 1987; Tischler *et al.*, 1986; Van Nguyen *et al.*, 1990). Together these models allowed several potentially important regulatory motifs to be identified (Morrison *et al.*, 1994a; 1994b; Mendelson *et al.*, 1995; Paterson *et al.*, 1995a, 1995b, 1995c). The PPT-A promoter has been shown to contain many activator and repressor binding sites that may function *in vivo* to allow tissue specific and stimulus-inducible expression of the PPT-A gene. The important regulatory elements that have been determined are summarised in figure 1.4.

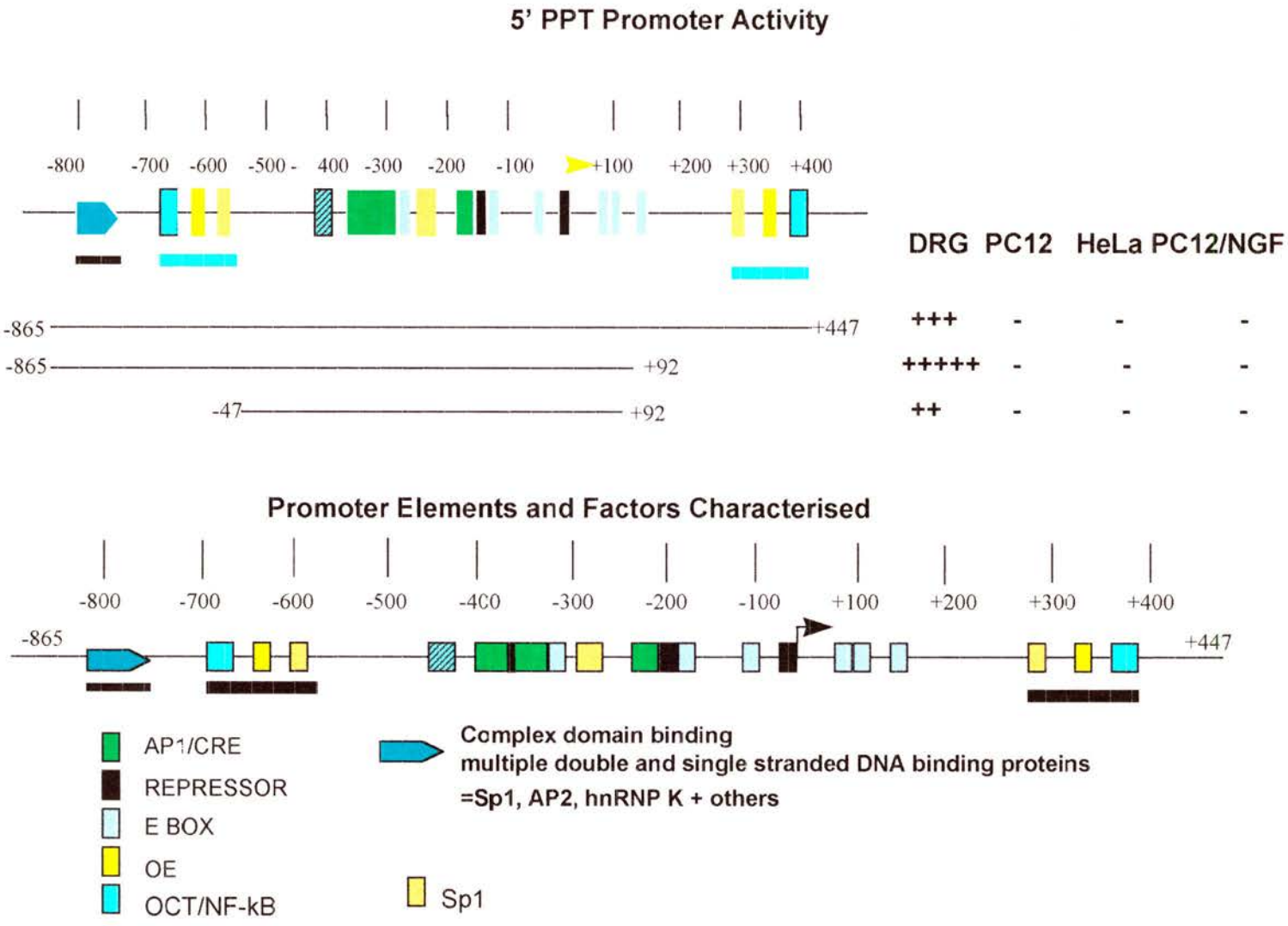

Figure 1.4: Diagram of transcription factor interactions and summary of previously characterised promoter fragment activity in DRG cultures and PC12 and HeLa cell lines. See sections 1.2.3.1 and 1.2.3.2 for details

1.2.3.1 Repression of the PPT-A promoter

It is known that negative regulatory elements are present in neuronal-specific genes (Hoyle *et al.*, 1994; Maue *et al.*, 1990; Mori *et al.*, 1990; Mori *et al.*, 1992). The promoter domain –865+92 can drive high levels of reporter gene expression in DRG neurons but not in clonal cell lines HeLa and PC12 (Mendelson and Quinn, 1995). This indicated that *cis*-acting elements may be regulated by neuron specific factors or that PPT-A promoter activity is repressed in non-neuronal cells. DNase1 footprinting demonstrated that the PPT-A promoter bound a sequence specific complex between the TATA box and the major transcription initiation site. This was believed to be a dominant repressor that could potentially interfere with the binding of the basic transcription machinery and could therefore account for neuron-specific expression of the PPT-A promoter. Reporter gene constructs containing the PPT-A promoter spanning base pairs –3356 to +92 were co-transfected into HeLa cells with increasing concentrations of a plasmid, containing multiple copies of the potential repressor binding site. PPT-A promoter activity was observed in the presence of high levels of the repressor sequence suggesting that the multiple copies of the repressor binding site acted to titrate the repressor from the PPT-A promoter allowing expression (Mendelson and Quinn, 1995).

In addition to this dominant repressor, other elements have been associated with exerting negative effects on PPT-A promoter activity. The region spanning nucleotides –198 to – 155 contains both an AP1/CRE element in addition to an E box motif. Originally it was shown that the AP1/CRE motif alone (spanning nucleotides –198 to –182) could activate reporter gene expression in DRG neurons and in HeLa and PC12 cell lines, when linked to a heterologous minimal promoter. In addition,

this potential AP1/CRE element was found to preferentially bind a protein complex that is distinct from CREB, ATF1, Fos and Fra. (Morrison *et al.*, 1994a). Later when the entire region spanning –198 to –155, incorporating the AP1/CRE elements together with the E box motif, were similarly examined, it was established that this fragment was inactive in HeLa and PC12 cells but could support reporter gene expression in DRG cultures. This indicated the presence of a second repressor element associated with nonneuronal cells which was acting to repress the previously identified AP1/CRE enhancer element in PC12 and HeLa cell lines (Paterson *et al.*, 1995c).

Recently, clonal cell lines RINm5F (McGregor *et al.*, 1992) and NF2C cells (Whitehead and Joseph, 1994) have been found to express endogenous PPT-A and support expression of the PPT-A promoter (Fiskerstrand *et al.*, 1999). The RINm5F cells have been derived from a rat pancreatic tumour and NF2C cells were generated from a transgenic rat in which a temperature-sensitive SV40 LT antigen is expressed under the control of the neurofilament light-chain promoter (Kilty *et al.*, 1999). These cell lines allowed functional analysis of putative regulatory elements in the promoter and the identification of a novel repressor domain lying 3' of the major transcriptional start site (Fiskerstrand *et al.*, 1999). Cells were transfected with reporter constructs containing PPT-A promoter fragments driving the luciferase reporter gene. Comparison of plasmids containing promoter fragments –865+447 and –865+92 showed that sequences located between +92 and +447 were exerting negative regulatory effects in both cell lines and DRG neurons (Fiskerstrand *et al.*, 1999). This effect was shown to be due to sequences located between base pairs +367 to +396 as mutation of this site relieved repression of the –865+447 promoter

fragment. This region has been characterised as having the potential for forming multiple DNA binding complexes and contains binding sites for transcription factors Oct-1 and NFκ-B (Mendelson and Quinn, 1995; Mendelson *et al.*, 1998; Fiskerstrand *et al.*, 2000). Mutation of the octamer binding site in this region was not associated with loss of protein binding but in functional studies did result in alleviation of repression (Fiskerstrand *et al.*, 2000). Therefore loss of repression following mutation of this octamer binding site is not correlated with loss of octamer binding to this region.

1.2.3.2 Activation of the PPT-A promoter

Under specific circumstances, the PPT-A promoter fragment –865+92 can be active in PC12 cells. Following forskolin treatment in combination with potassium evoked depolarisation, promoter activity was observed (Morrison *et al.*, 1994b). Forskolin acts to induce members of the cAMP response element binding family of transcription factors (CREB) (Montiminy *et al.*, 1986) whereas depolarisation causes the induction of members of the AP1 family of transcription factors (Bartel *et al.*, 1989). The induction of the PPT-A promoter by forskolin and potassium evoked depolarisation in PC12 cells was thought to be associated with the presence of an E box motif located between nucleotides –67 to –47. When this region was mutated, expression in PC12 cells was no longer observed (Paterson *et al.*, 1995b). DNase1 footprinting had previously identified protein complexes binding to this element (Mendelson *et al.*, 1995; Mendelson and Quinn, 1995) and it was shown to bind the bHLH transcription factors USF and max (Paterson *et al.*, 1995b). In addition, constructs containing this fragment –67 to –47 could drive reporter gene expression

in HeLa and PC12 cells (Mendelson *et al.*, 1995). When this construct was used for transfection of HeLa and PC12 cells and exposed to TPA and NGF treatment respectively, promoter activity was increased. When it was microinjected into DRG neurons, NGF had no effect on the activity. As previously stated, this was thought to be due to the overlap of signal transduction pathways activated by the process of microinjection in addition to NGF.

The region spanning nucleotides –345 to –308 was shown to contain two AP1 sites adjacent to an E box binding consensus. The AP1 sites in this region were shown to bind a complex that was recognised by antibodies to c-fos/fra. When these three elements were analysed individually and together, they were shown to behave differently. When the individual AP1 elements were linked to a heterologous promoter to drive reporter gene expression no activity was observed in PC12 cells. However high levels of activity were observed when all three elements were provided together, indicating that these elements may act synergistically to regulate PPT-A promoter activity (Paterson *et al.*, 1995c).

Another octamer binding site located 5' of the major transcriptional start site (situated –567 to –546) has been shown to be important for positively regulating the PPT-A promoter. Mutation of this site resulted in both loss of promoter activity in reporter gene analysis and loss of protein binding in EMSA, confirming enhancer function of this 5' octamer motif (Fiskerstrand *et al.*, 2000). This 5' octamer binding element is bound by the same classes of transcription factors as the 3' octamer motif described previously (section 1.2.3.1, Fiskerstrand *et al.*, 1999) and it is thought these domains may act synergistically to regulate PPT gene expression. As described in section 1.2.2.3, octamer binding proteins have been implicated in the regulation of

neuron-specific gene expression and in mediating the effects of NGF on sensory neuron gene expression (Wood *et al.*, 1992).

DNA binding studies have identified other potential regulatory elements, however functional analysis of these specific elements has not yet been performed. These include the identification of purine-rich motifs located between nucleotides –284 to –264, –576 to –546 and –779 to –775 (Quinn *et al.*, 1995; Mendelson and Quinn, 1992; Quinn and McAllister, 1993). The region spanning –779 to –775 has been shown to specifically interact with AP2, Sp1, a sequence specific single-stranded DNA binding protein and other complexes (Quinn *et al.*, 1995). These elements may serve as binding sites for AP2, Sp1 or other zinc finger containing proteins in neurons. In addition to the three E box motif described previously, a further three E box motifs have been identified 3' of the transcription start site on the PPT-A promoter. These are located at nucleotides +61 to +66, +69 to +74 and +181 to +186 (Mendelson and Quinn, 1995). Functional analysis of these E box motifs is still required to determine if these are important elements in regulating PPT-A promoter activity.

1.2.3.3 Stimulus inducibility of the PPT-A promoter

Transcription factors stimulate or repress gene expression in response to extracellular stimuli delivered to the cell membrane by, for example, hormones, neurotransmitters or growth factors. The mechanisms that link the cell membrane to the nucleus involve the activation of signal transduction pathways in which the final step involves the activation or binding of transcription factors to specific DNA elements on the gene promoter (figure 1.5). NGF is known to increase PPT-A mRNA

and SP expression levels in DRG neurons (Lindsay and Harmar, 1989; Vedder *et al.*, 1993; Mulderry, 1994; Jiang and Smith; 1995; Leslie *et al.*, 1995; Otten and Lorez, 1983) and therefore is likely to regulate SP expression at the levels of transcription.

The PPT-A promoter is known to contain putative binding sites for transcription factors that are also induced in response to NGF. Factors include AP1, CREB, octamer binding proteins and the bHLH families of proteins, which have been shown to be regulated by NGF (Curran and Franza, 1988; Curran and Morgan, 1985; Ginty *et al.*, 1994; Milbrandt, 1986; Quinn, 1991; Wood *et al.*, 1992 and Mendelson *et al.*, 1992). The bovine PPT-A promoter region has been shown to contain elements that can mediate the effect of NGF in PC12 and F11 cell lines (Gilchrist *et al.*, 1991a, 1991b and 1992). Comparison of the 5' flanking region of the rat PPT-A gene with that of the bovine and human shows that the sequences are highly similar between base pairs –750 and +1 but diverge considerably upstream of -750 (Chapman *et al.*, 1993). It might be expected that the sequences essential for neuron-specific and inducible expression are located within this conserved region.

Recently, using adeno-associated virus (AAV) as a tool for transduction of cultured DRG neurons, it was shown that exogenous NGF induced expression of the PPT-A promoter (Harrison *et al.*, 1999). In addition to NGF, other growth factors have been implicated in regulating neuropeptide levels in DRG neurons. Like NGF, they may be acting at the level of transcription and therefore may be capable of regulating PPT-A promoter activity. The growth factors that can regulate DRG phenotype and may have a role in mediating plasticity of the PPT-A gene in DRG neurons by acting at the level of transcription will now be discussed.

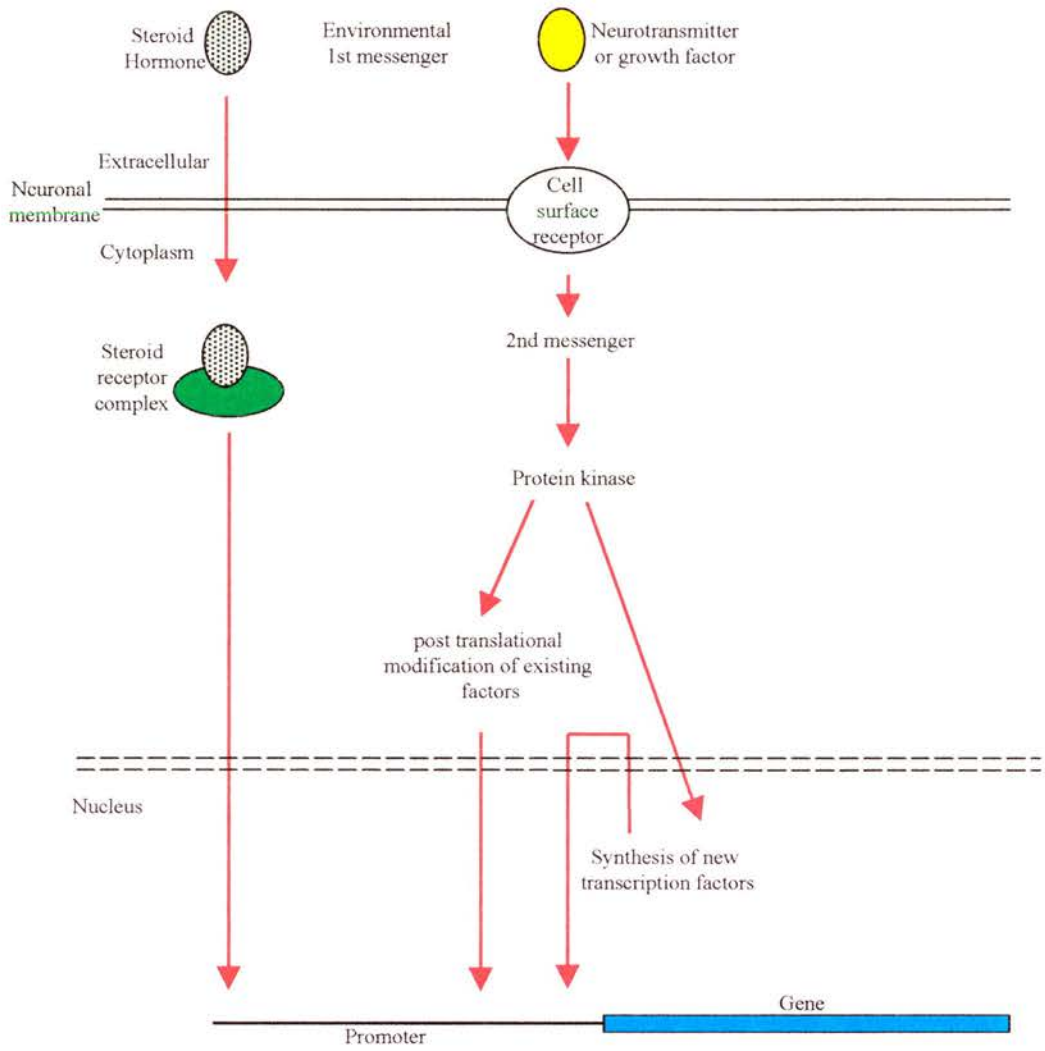

Figure 1.5: Signal regulated transcription factors mediate changes in gene expression within the neuron. Changes in gene expression are controlled by signal-regulated transcription factors (TF) which include ligand-activated TFs of the steroid hormone family, post-translationally activated TFs e.g. CREB family and transcriptionally activated TFs e.g. c-fos, c-jun.

1.3 Dorsal root ganglia (DRG) neurons and neurotrophins

Cultures of DRG neurons are commonly used for investigating sensory neuron physiology and neuronal plasticity. However interpretation of the data can be difficult due to the heterogenous nature and complexity of the DRG. Cultures obtained from DRG could represent more than 25 diverse populations that are specialised to generate a variety of sensory events (Petruska *et al.*, 2000). During development different populations are dependent on different growth factors and these are essential for the generation of neuronal heterogeneity. Although adult DRGs do not require these neurotrophins for survival, they do seem to require them for the continuous maintenance of mature phenotypic characteristics (Crowley *et al.*, 1994; Conover *et al.*, 1995; Sendtner *et al.*, 1996). To understand the role of growth factors and neurotrophins in adult sensory neurons, DRG neurons have been extensively studied and various populations classified according to morphology, anatomical location and electrophysiological and biochemical properties (Lawson, 1992). In particular, neurotrophic molecules influence distinct but overlapping subsets of neurons and populations are defined by differences in their responsiveness to these growth factors (Snider, 1994). Neuronal growth factors play an important role in influencing neuronal phenotypes and therefore in conducting sensory information. They function by binding and activating specific receptor tyrosine kinases. This then triggers a cascade of events that culminates in specific programmes of gene expression and cellular responses. This section will first briefly summarise the neurotrophins that have been associated with sensory neurons and regulating PPT-A expression (table 1.1). Subsequently, different neuronal populations of the DRG will be discussed since this is relevant to the regulation of

tachykinins in DRG neurons. Different populations of sensory neurons contain different neurotrophin receptors and therefore neuropeptide expression may be differentially regulated between these cell populations.

1.3.1 Neurotrophic factors and their receptors

The neurotrophin family of neuronal growth factors includes nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin- 4/5 (NT-4/5). Neurotrophins trigger a variety of biological responses including enhanced neurite outgrowth (Cohen *et al.*, 1994; Segal *et al.*, 1995), changes in electrophysiological properties of neurons (Kalman *et al.*, 1989; Levine *et al.*, 1995) and alterations in neuronal cell fate (Sieber-Blum, 1991).

Neurotrophins are produced by target tissues and, upon their release, bind to specific receptors located on the surface of peripheral axons. Each neurotrophin is capable of interacting independently with the widely distributed low affinity receptor p75 and specific Trk receptor kinases. The Trk receptors display neurotrophin selectivity with the TrkA, TrkB and TrkC tyrosine kinases serving as the receptors for NGF, BDNF and NT-3 respectively, and TrkA and TrkB can also act as receptors for NT-3 and NT-4/5. P75 is thought to modulate the sensitivity of cells to different neurotrophins (Chao and Hempstead, 1995), however in the absence of Trk receptors, neurotrophins binding p75 can lead to cell death (Meldolesi *et al.*, 2000).

NGF is the prototypic neurotrophin therefore its mode of action and resulting effects have been extensively studied (figure 1.6) (for review see Segal and Greenberg, 1996). It is known that dimeric NGF molecules bind to TrkA and causes the formation of TrkA dimers (Jing *et al.*, 1992). This in turn results in internalisation

and the receptors are transported to the soma. In addition, cross-linking leads to phosphorylation of the TrkA molecules, which catalyses the formation of large signalling complexes through the recruitment of cytosolic and membrane associated protiens (Heldin, 1995). The final step of this cascade involves phosphorylation of transcription factors that will act directly or indirectly to regulate gene expression (Stephens *et al.*, 1994). As stated previously NGF is important for neuropeptide plasticity in DRG neurons and can increase SP expression. This effect is mediated, at least in part by regulatory elements located on the PPT-A proximal promoter and loss of NGF as in axotomy is thought to account for the characteristic decrease in SP expression following nerve injury.

Neurotrophin glial cell-derived neurotrophic factor (GNDF) has been shown to alter sensory neuron phenotype. GDNF is a member of a novel family of trophic factors that also include neurturin, persephin and artemin and can exert notable trophic influences on dopaminergic neurons (Tomac *et al.*, 1995), motor neurons (Henderson *et al.*, 1994; Yan *et al.*, 1995) and sensory neurons (Matheson *et al.*, 1996). Members of the GDNF family exert their effects via a unique multicomponent receptor complex. This consists of Ret, a tyrosine kinase receptor acting as a signal transducing domain, in combination with a member of the GFRα family of the glycosyl-phosphatidyl-inositol-linked GDNF-family receptors (GFRαs), acting as a ligand binding domain (Airaksinen *et al.*, 1999; Durbec *et al.*, 1996; Treanor *et al.*, 1996).

Immunocytochemistry has identified the presence of GDNF in DRG sensory neurons (Hostelge *et al.*, 1998) and GDNF mRNA has been shown to be rapidly up-regulated in DRG neurons, satellite cells, and in schwann cells following nerve

transection (Hammarberg *et al.*, 1996; Bar *et al.*, 1998; Kashiba *et al.*, 1998; Hoke *et al.*, 2000). GDNF is thought to have a role in normal and injured adult peripheral nerves. Recently it has been shown that SP levels are increased in cultured DRG neurons following treatment with exogenous GDNF (Adler, 1999; Ogun-Muyiwa *et al.*, 1999). It is therefore thought that GDNF, like NGF, might regulate PPT-A expression at the level of transcription.

In addition to the NGF and GDNF related families of neurotrophins, other molecules are known to exert trophic effects on DRG and are involved in regulation of PPT-A gene expression. These include members of the neuropoeitic group of cytokines, leukemia inhibitory factor (LIF), interleukin–6 (IL-6) and ciliary-derived neurotrophic factor (CNTF). These proteins have multiple actions on cells of the nervous system. These include promoting the survival of some neurons (Pennica *et al.*, 1995; Stahl and Yancopoulos, 1994) and regulating neuropeptide expression in cultured sympathetic neurons (Nawa *et al.*, 1991; Zigmond and Sun, 1997; Fann and Paterson, 1994). In addition, LIF is thought to function as a lesion-induced factor involved in the cell body reaction to axotomy in the sympathetic nervous system (Sun *et al.*, 1994; Zigmond *et al.*, 1996).

These molecules have been grouped as a family due to partially overlapping biological activities and are defined by their binding to the common receptor motif gp130 transmembrane glycoprotein. Two gp130 molecules form part of the IL-6 receptor, and one molecule together with another transmembrane glyoprotein termed the LIF receptor beta subunit (LIFRβ) are components of the receptors for LIF and CNTF. The CNTF and IL-6 receptors are additionally comprised of alpha subunits: CNTFRα and IL-6Rα (Horton *et al.*, 1998). The LIF, IL-6 and CNTF receptors are

constitutively associated with the members of the Jak-Tyk family of tyrosine kinases (Stahl *et al.*, 1994). The first step of the CNTF signalling pathway involves binding of CNTF to the α-receptor subunit, which promotes association and dimerisation of two β-receptor subunits. This results in activation of the associated Jak tyrosine kinases and propagation of the CNTF signal.

LIF and CNTF have overlapping effects (Ip and Yancopoulos, 1992) and in some cases can elicit similar biological responses to the NGF-related family of neurotrophins (Mitsumoto *et al.*, 1994), suggesting that the neurotrophin and cytokine signalling pathways may interact synergistically or may act to antagonise the effects of neurotrophins. LIF is thought to play a role in regulating neuropeptide levels in sensory neurons following axotomy and may be an important regulator of PPT-A gene expression.

Growth factor family	Members	Receptor
NGF related neurotrophins	NGF, BDNF, NT-3, NT-4/5	Trk tryrosine kinases and p75
GDNF-related neurotrophins	GDNF, neurturin, persephin, artemin	Ret tyrosine kinase (signal transducing domain) plus member of the GFRα family (ligand binding domain)
Neuropoeitic cytokines	LIF, IL-6, CNTF	gp130 transmembrane glyoprotein together with specific receptor subunits for each factor

Table 1.1: Summary of growth factor families and their receptors that may be involved in the regulation of PPT-A expression.

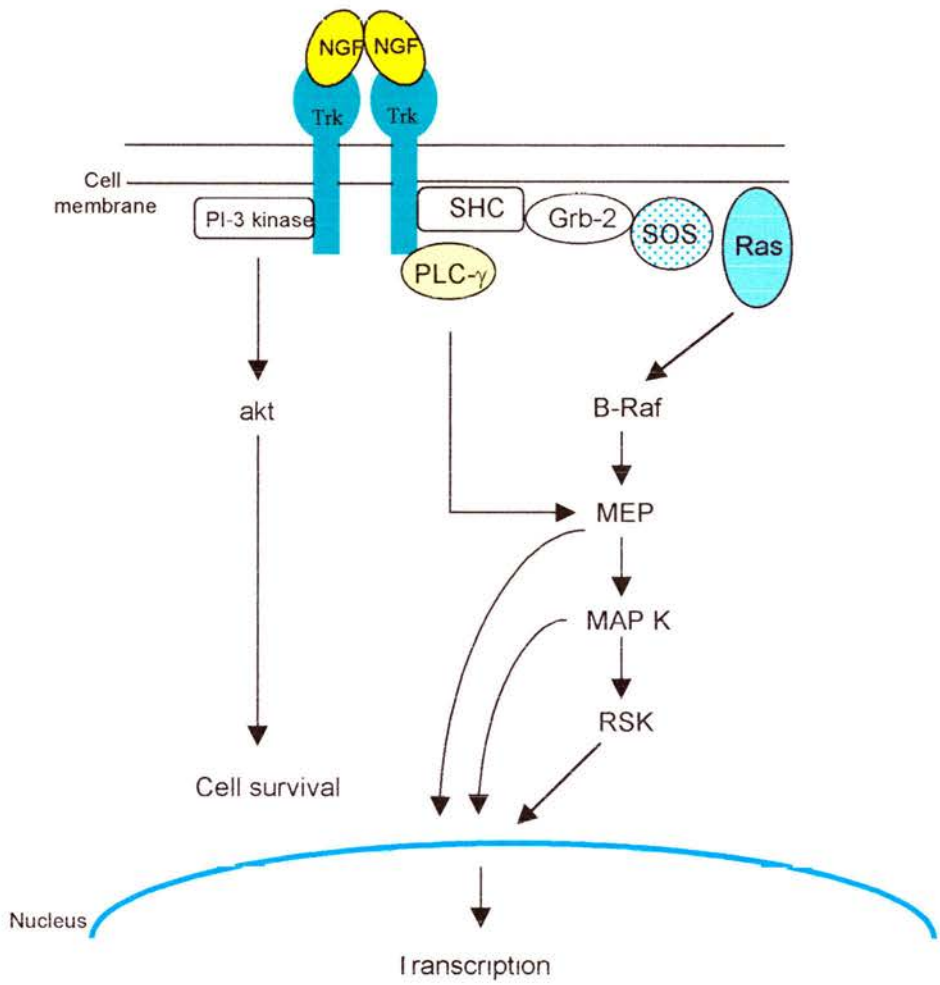

Figure 1.6: Trk signalling pathways. NGF binds to two trk molecules, causing the formation of a homodimer, which in turn permits each molecule to phosphorylate tyrosine residues on its partner. Phosphorylation of specific tyrosine residues creates signal binding sites for PI-3 kinase, PLC-γ and Shc, recruitment of these proteins into a complex and initiation of a signaling cascade.

1.3.2 Sensory neurons of the DRG

Sensory neurons of the DRG have been broadly divided into two main groups based on cell body size, termed small diameter dark and large light diameter population of neurons (table 1.2) (Lawson, 1992; 1996). These groups convey different types of sensory information. The large diameter neurons are myelinated (A fibres) and are thought to function as proprioceptors and mechanoreceptors. They posses cell diameters of greater than 30 μm and can be labelled using antibodies against the high molecular weight neurofilament protein (NF-H) (Averill *et al.*, 1995; Molliver *et al.*, 1995). This group comprise 15-20% of all DRG neurons and all express the NT-3 receptor TrkC (Wright and Snider, 1995).

Small neurons have a cell body diameter of less than 30 μm and are unmyelinated (C fibres). These are thought to function as nociceptors and can simplistically be further divided into two groups, those that express neuropeptides and the non-peptidergic expressing neurons. The peptide containing population express substance P (SP) and calcitonin gene related peptide (CGRP). These possess the NGF receptor TrkA and therefore the peptide content of these cells can vary in response to NGF.

The other subpopulation has been shown to switch its neurotrophin requirement postnatally from NGF to GDNF (Molliver and Snider, 1997; Molliver *et al.*, 1997; Bennett *et al.*, 1996; 1998a). Neurons of this group are neurochemically distinct from the population of NGF responsive neurons as they do not express CGRP and SP. This population can be labelled selectively by the isolectin B-4 (IB-4) and express the enzyme thiamine monophosphatase (TMP) (Averill *et al.*, 1995; Molliver *et al*,. 1995, 1997). Virtually all IB4 labelled cells express Ret and GFR-α,

the receptors for GDNF. (Bennett *et al.*, 1998b). C-ret, the functional receptor for GDNF, was also found to be present on some of larger, non-nociceptive A fibres (Honda *et al.*, 1999).

These three groups define broadly the populations of sensory neurons. However the situation is much more complex and co-expression studies have revealed the potential for overlapping populations of marker molecules. In particular, some studies report that distinct populations express only one Trk receptor (Mu *et al.*, 1993; Kashiba *et al.*, 1995; Wright and Snider, 1995) whereas others report more than one Trk receptor to be present in a single neuron (McMahon *et al.*, 1994; Karchewski *et al.*, 1999). It is believed that the presence of multiple neurotrophin receptors on a sensory neuron allows it to respond to a variety of neurotrophins. This exposes the potential for convergent or divergent signalling pathways. In addition, expression of growth factor receptors can be altered in response to nerve damage (Krekoski *et al.*, 1996; Cho *et al.*, 1996; Narita *et al.*, 2000) and arthritis (Pezet *et al.*, 2001) illustrating the complexity and plasticity of these growth factors associated with DRG neurons.

Properties	**Class of DRG neuron**		
	1	**2**	**3**
Size of cell body	> 30 μm	< 30 μm	<30 μm
Myelinated/unmyelinated axons	Mylinated	Unmylinated	Unmylinated
Neurotrophin Receptors	TrkC mainly Some TrkB and TrkA	TrkA	C-RET
Function	Mechanoreceptors and proprioceptors	Nociceptive	Nociceptive
Growth factor responsive	BDNF and NT-3	NGF	GDNF
Markers	Neurofilament	CGRP/SP	IB-4

Table 1.2: Summary of the broadly defined populations of DRG neurons.

1.4 Use of adeno-associated virus to investigate transcriptional regulation of the PPT-A promoter

The studies outlined in section 1.2 have revealed a great deal about the factors that may be involved in regulating PPT-A promoter activity, however as discussed previously functional analysis has been difficult. The recent discovery of cell lines which support the expression of reporter gene constructs directed by the PPT-A promoter should prove to be a useful tool for investigating PPT-A transcriptional regulation. However, there is still difficulty with studying promoter activity in DRG neurons and as described in section 1.2.3, experiments have revealed there may be differences between the activity of regulatory elements in DRG neurons and cell lines. Previous experiments investigating promoter activity in DRG neurons employed the technique of microinjection of plasmid DNA into DRG neurons. This is extremely time-consuming, laborious and allows the analysis of only a small number of neurons. In addition, Quinn *et al.* (2000) suggested that the process of microinjection might activate stress signal transduction pathways, which may overlap with those activated by NGF and other growth factors. Recently our group has used AAV for gene transfer into DRG neurons. These experiments have revealed that the PPT-A promoter fragment –865 to +92 contains regulatory elements that can be induced by NGF when the promoter is delivered as a recombinant AAV (rAAV) particle (Harrison *et al.*, 1999). This result was in stark contrast to the microinjection data and therefore this model system will be suitable for investigating the effect of other growth factors described in section 1.3.1 on the PPT-A promoter, in addition to defining potential transcription regulatory elements.

Interest in the ability to exploit rAAV vectors for gene therapy and experimental manipulation of the nervous system has encouraged investigation into

the unique biology of this virus. Understanding the biology of AAV has provided the foundations for many developments of AAV as a DNA delivery vector. Therefore the biology of wild-type AAV (wtAAV) will first be outlined before discussing the work that has focused on improving the rAAV vector system.

1.4.1 Wild-type adeno-associated virus (wtAAV)

Adeno-associated virus (wtAAV) is a member of the parvoviridae family, which are among the smallest and structurally simplest of the DNA animal viruses. Parvoviruses are nonenveloped, icosahedral with linear single-stranded genomes of 4.6 – 6 kb. AAV was initially believed to be a defective virus since co-infection with a helper virus (usually adenovirus or herpes simplex virus) is necessary for replication and in helper absence AAV may be maintained latently. The helper functions required for productive infection have been best characterised for adenovirus coinfection. With the exception of E2b and E3, all of the adenovirus early functions (E1a, E1b, E2a and E4) contribute to helper function in cell culture and effect gene expression, both viral and cellular (Richardson and Westphal, 1981).

Cell damage due to chemical mutagens, ultraviolet irradiation and other cellular insults can also induce lytic life cycle (Yakinoglu *et al.*, 1988; Yakobson *et al.*, 1987). Five serotypes of AAV have been identified, the most characterised being AAV-2.

1.4.2 AAV genetics

The wtAAV genome is 4.7 kb and consists of two open reading frames (ORFs), the 5' and 3' ORF (figure 1.7). The 5' ORF encodes four regulatory

proteins, Reps 78, 68, 52 and 40, which are important for gene expression and DNA replication. The 3' ORF encodes the capsid proteins, Vp1, Vp2 and Vp3. There are three promoters: two in the 5'ORF at map positions (mp) 5 and 19 (promoters p5 and p19 respectively) and one in the 3' ORF at mp 40 (promoter p40). Spliced and unspliced transcripts initiated from p5 and p19 are translated to generate all four Rep proteins. The capsid proteins are translated from a transcript whose promoter is p40 and are generated as a result of alternative mRNA splicing. Vp1 is translated from the minor species and Vp2 and Vp3 from the more abundant mRNA. Vp3 is translated from the first in-frame initiator codon in the major mRNA species whereas Vp2 is initiated from an upstream, in-frame ACG codon (Berns, 1990).

An important feature of the AAV genome is the presence of the 145 nucleotide inverted terminal repeats (ITR). The first 125 bases of these can form a T-shaped hairpin structure that is composed of two small inverted palindromes, flanked by a larger palindrome. Like the Rep proteins, the ITRs are involved in every step of the AAV life cycle: regulation of gene expression, regulation of DNA replication, site-specific integration and rescue of the genome from the integrated state (Kotin *et al.*, 1992; Xiao *et al.*, 1997).

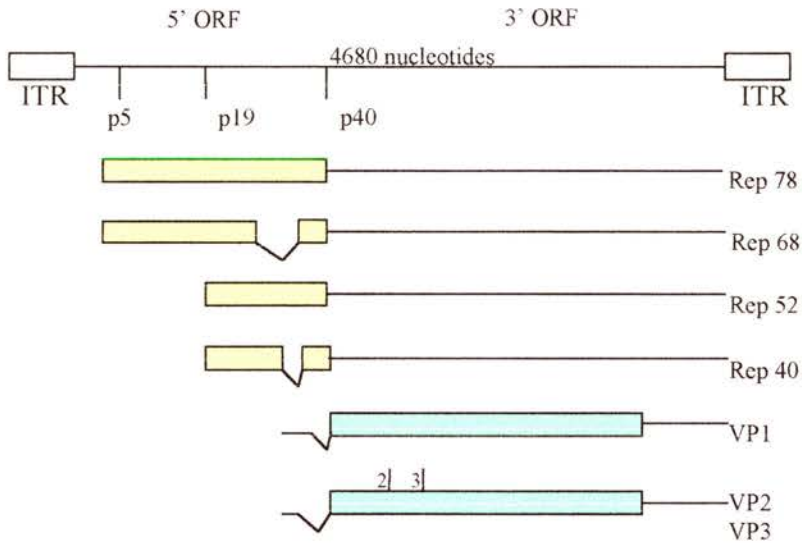

Figure 1.7 : Genetic map of AAV. Promoter at map position 5 (p5) initiates a transcript which, unspliced encodes Rep 78 and spliced encodes Rep 68. Promoter p19 at map position 19 encodes a transcript that is translated intact, yielding Rep 52 or spliced, to yield Rep 40. p40 initiates all three capsid mRNAs. Vp1 is produced by an alternative splicing event, Vp2 is produced by the same spliced event used for Rep proteins (Rep 68 and 40). Utilising the same mRNA as Vp3, Vp2 results from using an alternative ACG start codon (from Berns and Linden, 1995).

1.4.3 AAV productive infection

In the presence of a helper virus, wtAAV undergoes productive infection (figure 1.8). The virion penetrates to the nucleus of the cell and the virus genome is uncoated. Two elements are essential for wtAAV DNA replication, the viral origin of replication that is located within the 145 base terminal repeats and the *rep* gene. Mutation analysis has shown that Rep 78 and 68 proteins are essential for replication (Labow and Berns, 1988) and regulation of AAV gene expression (Labow *et al.*, 1986; Beaton *et al.*, 1989). Rep 68/78 has been shown to possess DNA binding, site-specific endonuclease and helicase activities (Im and Muzyczka, 1990). It is unclear what roles the smaller proteins Rep 52 and 40 have, although they are thought to function in virus assembly (Chejanovsky and Carter, 1989). As noted previously, the function of adenovirus is also necessary for wtAAV replication. In the absence of adenovirus, Rep 68/78 is a negative regulator of wtAAV gene expression.

The palindromic ITR can act to initiate DNA replication by forming a hairpin primer allowing DNA synthesis by a single-stranded displacement mechanism. This results in the generation of a double-stranded replicative intermediate that is covalently joined at one end by this palindromic hairpin (Straus *et al.*, 1976). This structure is then resolved by nicking of the hairpin on the parental strand by Rep 68/78 directly opposite the 3' terminus. The result of this process is to leave a 3' OH on the parental strand. This then serves as a primer for synthesis to fill in the gap region and the hairpin sequence is transferred to the daughter strand and can then act as a template (for review see Berns, 1990).

1.4.4 AAV latent infection

Under non-permissive conditions, wtAAV may integrate into the host genome where it is maintained as a latent provirus (figure 1.8). In humans, the wtAAV genome will preferentially integrate into the q-arm of chromosome 19 at a site termed AAVS1 (Kotin *et al.,* 1990, 1992; Samulski *et al.,* 1991). The only homology between the integrated wtAAV DNA and the flanking cellular sequences is a 1-5 base overlap at the junction. Like DNA replication the process of integration requires the ITRs and Rep 68/78 (Balague *et al*., 1997; Surosky *et al*., 1997). The ITRs are essentially the only *cis*-acting elements necessary for integration, however in the absence of the Rep proteins wtAAV may integrate randomly at low frequency (Xiao *et al.,* 1997). The mechanism for wtAAV integration is unclear however models have been proposed (Lindel *et al*., 1996; Rizzuto *et al*., 1999; Tsunoda *et al*., 2000). It is thought Rep 68/78 can bind a specific site on the ITR of the wtAAV genome and the AAVS1 site on host DNA. This initiates a complex process whereby recombination and integration occurs. In the absence of integration wtAAV genomes may be maintained as head-to-tail concatamer (Duan *et al*., 1998). An understanding of the process of integration and rescue of the wtAAV genome will be extremely important in the generation of future wtAAV vectors.

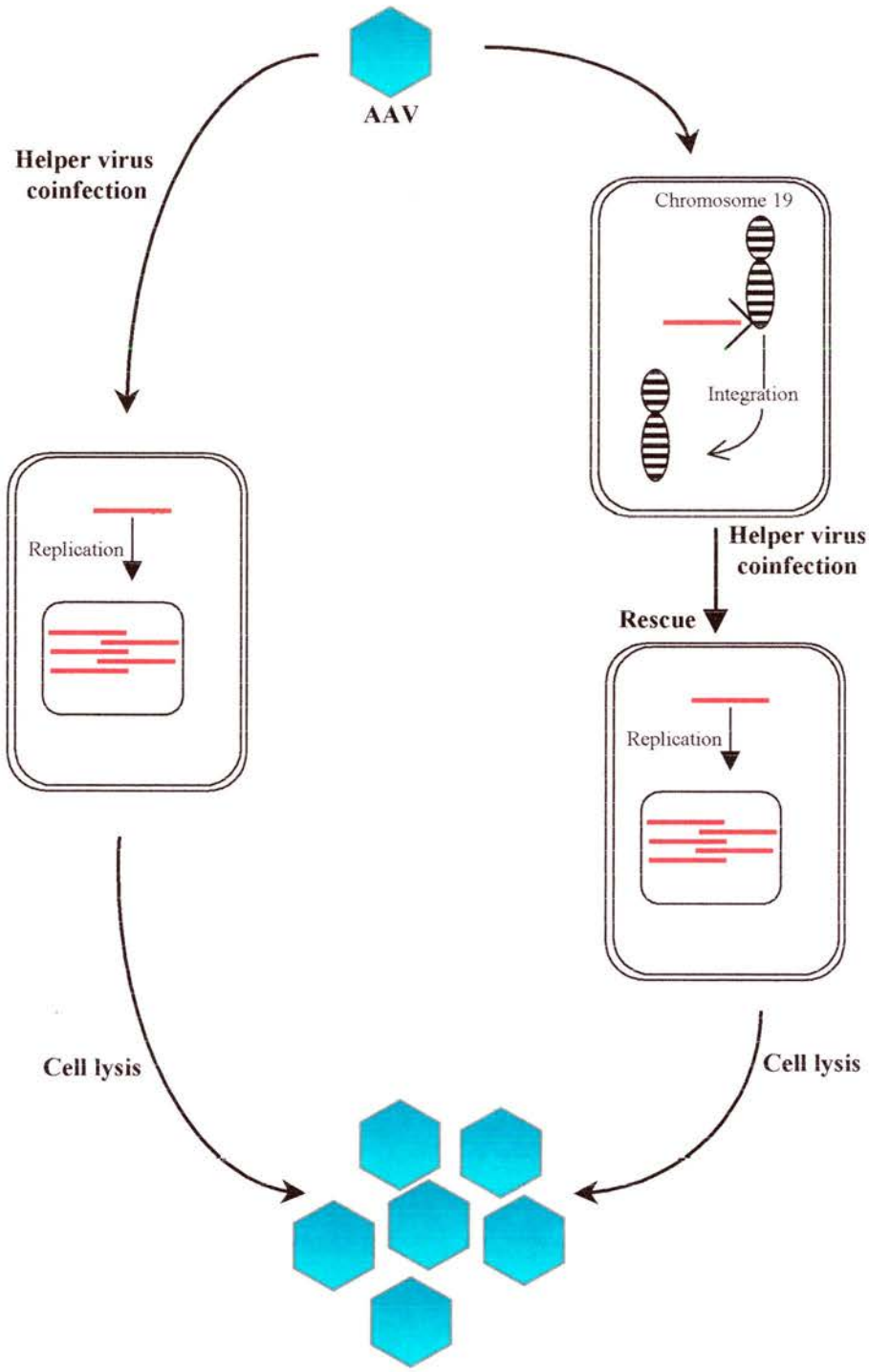

Figure 1.8: Model for AAV infection. Under non-permissive conditions, AAV integrates into the q-arm of human chromosome 19, where it remains silent until challenged by a helper virus. This leads to rescue of the integrated virus from the chromosome and induction of the lytic cycle. Under permissive conditions (in the presence of helper virus), AAV replicates resulting in host cell lysis.

1.4.5 AAV receptors and cell entry

Viral receptors are often involved in defining the host range and specific tissue tropism of a virus. Recently it has been shown that the cell surface heparin sulphate proteoglycan (HSPG) serves as the primary attachment receptor for wtAAV (Summerford and Samulski, 1998). Fibroblast growth factor receptor and $\alpha_v\beta_5$ integrin have also been implicated as coreceptors or facilitators (Qing *et al.*, 1999; Summerford *et al.*, 1999).

The mechanism of wtAAV entry has been examined and a model for wtAAV infection of cells proposed. Following binding to heparin sulphate receptors on the cell surface, wtAAV is internalised by endocytosis. This process is mediated by clathrin coated pits and the $\alpha_v\beta_5$ integrin receptor. Virus particles are then released into the cytosol and translocated to the nucleus where uncoating, replication and gene expression occurs (Bartlett *et al.*, 2000). A greater understanding of this early step in wtAAV infection will be important for understanding transduction of different cell types.

1.4.6 AAV vectors

There are a number of features that make rAAV vectors both an attractive tool for both neuron-specific expression studies (*in vivo* and *in vitro*) and therapeutic gene delivery. The safety features associated with this viral vector system are often highlighted in the hope that rAAV may be clinically beneficial for gene delivery, particularly the fact that rAAV has never been implicated as the causative agent for disease in humans (Berns and Bohenzky, 1987).

The methods employed to generate recombinant virus allow the production of rAAV particles without the risk of wild-type helper virus contamination (Samulski *et al.*, 1989) and eliminates the possibility of an immune response caused by viral gene expression (Yang *et al.,* 1994, 1995; Xiao *et al.*, 1996; Jooss *et al.,* 1998). Furthermore, rAAV viral particles are very stable, resistant to many physical and chemical factors such as various detergents, wide range of pH, certain proteases, repeated freeze-and-thaw cycles and heat denaturation up to 60 °C (Muzyczka, 1992).

wtAAV can infect both dividing and non-dividing cell types and rAAV vectors have been shown to transduce a variety of cell types. Delivery and long-tem expression of rAAV vectors has been shown in lung (Flotte *et al.,* 1994; Halbert *et al.*, 2000), muscle (Xiao *et al.,* 1996; Fisher *et al.,* 1997; Kessler *et al.*, 1996; Clark *et al.*, 1997), blood (Herzog *et al.*, 1999; Snyder *et al.*, 1999), cartilage (Pan *et al.*, 1999), brain (Bosch *et al.*, 2000; Lo *et al.*, 1999; McCrown *et al.*, 1996; Haberman *et al.*, 1998; Kaplitt *et al.*, 1994) and the peripheral nervous system (Glatzel *et al.*, 2000).

This ability of rAAV to introduce foreign genes into neuronal cell types is particularly important from an experimental point of view, allowing accessibility and manipulation of the PNS and CNS *in vivo*. This together with the *in vitro* transduction of neurons may now allow investigation of the fundamental questions regarding neuron specific gene expression.

1.4.7 AAV vector production

wtAAV has several biological properties that would make it a useful vector for gene therapy therefore there has been increased effort to develop AAV as a vector. The standard protocol for the production of rAAV vectors involves transient transfection of HEK293 (human embryonic kidney) cells with an AAV plasmid substrate and a helper plasmid (Samulski *et al.*, 1989), followed by subsequent infection with mutant adenovirus type 5, which lacks the E1a and E1b genes, (figure 1.9). The E1a and E1b genes are essential for replication of adenovirus and therefore the HEK293 cells have been transformed with the adenovirus genes E1a and E1b to provide these essential products, limiting the growth of adenovirus to the 293 cells.

Many studies employ the rAAV plasmid pSub201 (Samulski *et al.*, 1989) for the generation of rAAV vectors. Digestion of this plasmid with XbaI removes the wtAAV Rep and Cap sequences that are replaced by the desired gene but retains the wtAAV 145 bp ITRs flanking the gene of choice. These ITRs contain the recognition signals for DNA replication, packaging and integration of rAAV provirus and the rescue of integrated genomes. To produce the viral vector, the modified pSub201 plasmid is cotransfected with a helper plasmid containing the wtAAV rep and cap sequences (deleted from the pSub201 construct). This allows the AAV structural proteins to be provided *in trans*. Superinfection with adenovirus allows the replication of rAAV sequences, and single-stranded DNA molecules are then encapsulated into rAAV particles. rAAV particles are then released from infected cells by lysis, helper adenovirus particles are inactivated by heat treatment and rAAV vectors may be further purified by caesium chloride gradients. In this system the vector virions are free of contamination by wild-type AAV. Since Rep is required for

site-specific integration, it is uncertain whether rAAV vectors integrate randomly or mainly persist as episomes.

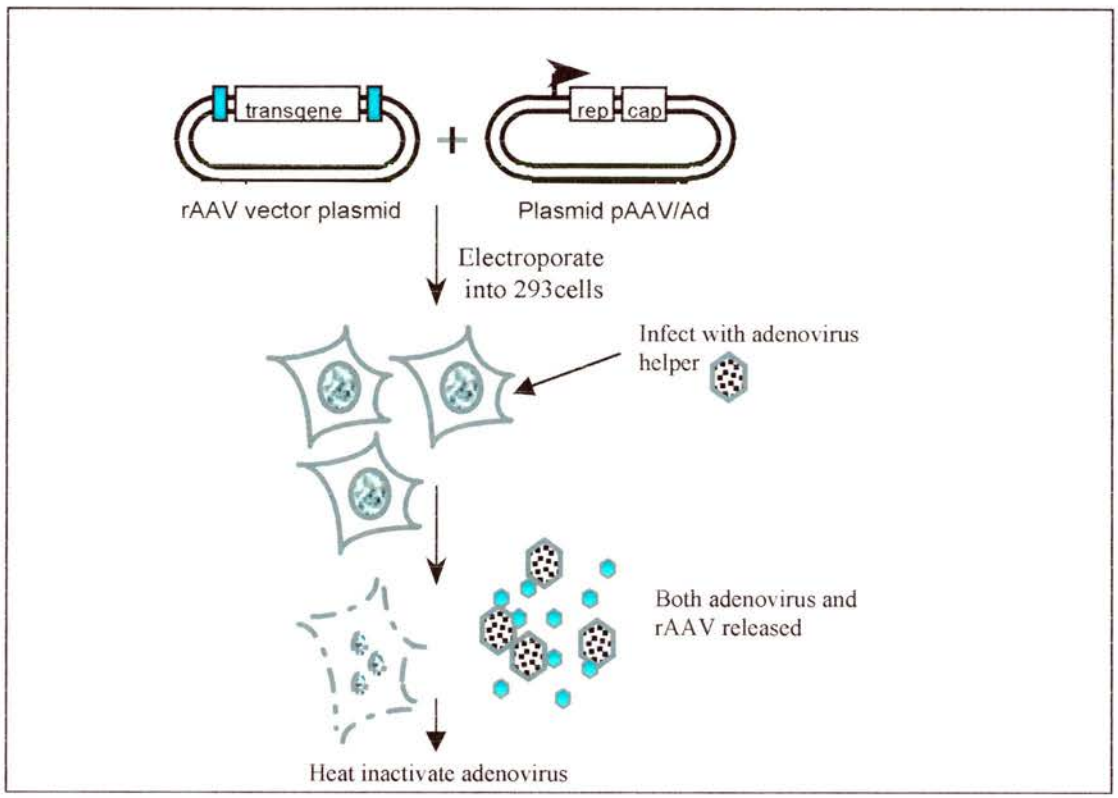

Figure 1.9: Standard protocol for the production of rAAV. 293 cells are co-transfected with rAAV helper plasmid and the AAV plasmid and are then infected with adenovirus. The AAVhelper plasmid pAAV/Ad provides the AAV genome *in trans* resulting in adenovirus and rAAV particle production. Both viruses are released and adenovirus is then inactivated by heating to 56 °C.

1.4.8 Limitations associated with AAV vectors

Although AAV has many positive attributes, there are some limitations associated with this virus system. One of the main difficulties associated with rAAV is the production of AAV preparations that have high titres, since AAV vectors are technically difficult to generate due to the lack of well-developed packaging cell lines. Other problems associated with use of AAV vectors include the size restraints associated with this system, as the maximum size of insert that can be used is 5 kb. In addition, the possibility of site-specific integration may be an important consideration if AAV vectors are to be used in gene therapy. However, the expansive research into the life cycle of AAV and improvements in AAV production are likely to overcome many of these drawbacks.

1.4.8.1 Improvements in rAAV production

The standard method for rAAV production described above (section 1.4.7) has formed the basis for generating rAAV vectors as a tool for both *in vitro* experiments and for gene therapy studies, however there are some limitations with producing rAAV vectors in this way. First, the fact that these preparations are contaminated with helper virus and must be purified leads to losses in the titre of rAAV. In addition, contamination with wild type AAV and residual adenovirus has been observed (Wang *et al.*, 1998; Xiao *et al.*, 1998) and this conventional protocol does not allow convenient large-scale production of vector particles. Therefore much research has been carried out to generate a system that overcomes these complications associated with rAAV production.

Recently, a production system has been developed which allows the generation of rAAV in the absence of helper adenovirus (Xiao *et al.*, 1998). A helper plasmid that contains elements of the adenovirus genome was constructed. This plasmid incorporates the essential helper genes, but lacks the adenovirus structural and replication genes, therefore is incapable of producing infectious adenovirus particles. Cotransfection of this adenovirus plasmid, together with the AAV helper plasmid and AAV packaging substrate, resulted in the generation of high titre rAAV particles. This approach has eliminated the possibility of unwanted adenovirus contamination. Other groups have created similar adenovirus helper plasmids (Matsushita *et al.*, 1998; Salvetti *et al.*, 1998). In addition, chimeric virus vectors which have AAV sequences together with adenoviral (Fisher *et al.*, 1996; Grimm *et al*,. 1998; Lieber *et al.*, 1999) and HSV (Conway *et al.*, 1999) sequences have been generated. The presence of AAV and helper sequences on one plasmid eliminates the need for the cotransfection of several plamids and therefore results in improved rAAV production and increased titres.

All of these alternative strategies have increased rAAV titre and decreased helper virus and wild-type AAV contamination. However, they do not allow the convenient scaling up of rAAV particles. To do this, the use of producer cell lines should allow bulk production of rAAV particles by overcoming the dependency on a transfection step. Initial efforts to develop cell lines containing AAV *rep* and *cap* genes was met with modest success and efficiency was poor with maximum rAAV yields not exceeding those of the basic protocol (Clark *et al.*, 1995; Tamayose *et al.*, 1996). Although eliminating the transfection step, these cells rely on the over expression of viral gene products Rep and Cap, which are toxic to cells. To date one

successful packaging system has been developed (Inque and Russell, 1998) which is based on inducible amplification of the integrated helper AAV constructs. Rep/Cap expressing cell lines have recently been used in conjunction with AAV-adenovirus hybrid vectors where the hybrid vector is used to deliver the rAAV genome to the packaging cell line in place of plasmid transfection (Gao *et al.*, 1998; Liu *et al.*, 1999).

1.4.8.2 Size limitations

rAAV vectors have a limited packaging capacity and transgenes to be delivered must be less than 5 kb and greater that 4.1 kb. Currently strategies are being explored to expand the AAV packaging capacity. These include the use of small promoters or promoter activity derived from the AAV ITR (Flotte *et al.*, 1993; Haberman *et al.*, 2000) and heterodimerization of separate rAAV vectors.

Heterodimerization of separate rAAV vectors takes advantage of a unique feature of wtAAV biology that results in head-to-tail concatenation of viral genomes during double-stranded conversion (Berns, 1996). Persistence of expression from rAAV vector occurs at least in part from latent AAV proviral DNA exisiting in head-to-tail concatamers, whether integrated or in nonintegrated circular form (Muzyczka, 1992; Xiao and Samulski, 1996; Duan *et al.*, 1998). Recently three separate groups have taken advantage of this inherent wtAAV feature and developed a strategy that bridges the 5' and 3' segments of a split expression construct through paired rAAV ITRs (Sun *et al.*, 2000; Yan *et al.*, 2000 and Nakai *et al.*, 2000). This means a larger candidate gene can be split into two parts and separately packaged into two individual rAAV vectors increasing the packaging capacity of AAV to 10 kb.

1.4.8.3 Strategies to eliminate the risk of insertional mutagenesis

rAAV vectors can integrate into the host genome, unlike wtAAV, it is not site-specific, and may therefore carry the risk of insertional mutagenesis. rAAV vectors have all the *cis*-elements necessary for integration, which can be carried out by host factors, but targeting is lost in rep-minus constructs (Yang *et al.*, 1997). The incorporation of a truncated Rep protein into a rAAV vector has been described recently, with the restoration of integration at the chromosome 19 site in tissue culture (Rinaudo *et al.*, 2000).

1.4.8.4 Improvements in rAAV gene transfer

Recent insights into wtAAV biology highlight the three main areas to be addressed to improve rAAV transuction: rAAV interaction with target receptors, efficient conversion to a double –stranded template and adequate expression from the rAAV-transgene construct.

Recently attention has been paid to the tropic nature of each of the five serotypes of wtAAV in order to improve transduction. The genomes of each of these wtAAV serotypes are organised similarly, however there is extensive heterogeneity within the surface exposed regions of capsid structures between the serotypes. This suggests that each serotype may have a distinct mechanism of cellular uptake (Chiorini *et al.*, 1999a; 1999b). Transduction efficiencies between serotypes was shown to differ between cell lines (Chiorini *et al.*, 1999a; 1999b) and regions of the mammalian brain (Davidson *et al.*, 2000). The elucidation of the mechanism of tissue tropism observed for the different wtAAV serotypes will contribute to designing rAAV-specific targeting vectors.

Other attempts to produce targeted rAAV vectors have focused on modifying the natural tropism of wtAAV by using cross-linked monoclonal antibodies to target wtAAV to antigens on the surface of non-permissive cells (Bartlett *et al.*, 1999) or by genetically altering the capsid coding region (Girod *et al.*, 1999, Rabinowitz *et al.*, 1999).

For efficient expression from rAAV vectors, virus must be converted from monomer single-stranded genome to double-stranded form and increased transgene expression levels are associated with the appearance of transgene copies in double-stranded DNA (Vincent-Lacaze *et al.*, 1999; Miao *et al.*, 1998; Malik *et al.*, 2000). Recently a mechanism for this conversion has been reported and a cellular Tyr phosphoprotein has been described which binds AAV ITRs and might regulate synthesis of the complementary strand (Qing *et al.*, 1998). It is thought that strategies to augment or accelerate the presentation of double-stranded template for transcription will increase transduction by rAAV vectors (Monahan and Samulski, 2000). High amounts of the dephosphorylated form of this protein have been shown to correlate with efficient transduction by rAAV (Qing *et al.*, 1998).

Persistence of expression from rAAV vectors depends on the generation of AAV concatamers and conversion to high molecular weight (HMW) DNA (Xiao *et al.*, 1996; Vincent-Lacaze et al., 1999). It is unclear why this is the case but an understanding of this would be extremely useful for ensuring long-term expression from rAAV vectors.

Long-term expression of transgenes has been increased by the addition of a post-transcriptional regulatory element. The woodchuck hepatitis virus

posttranscriptional regulatory element (WPRE) was shown to enhance the expression of a GFP reporter gene when delivered by a rAAV vector (Loeb *et al.*, 1999).

1.4.8.5. Improvements in rAAV purification

Improvements in virion purification have also been addressed. Purification of rAAV was initially performed by centrifugation over CsCl gradients, however this cumbersome technique yields preparations with a considerable amount of contaminating non-viral proteins and a high ratio of genome copies verses infectious units. Given that heparin sulphate proteoglycan is known to serve as the viral receptor for wtAAV, a heparin sulphate (HS) affinity column was used for rAAV purification (Clark *et al.*, 1999; Anderson *et al.*, 2000; Auricchio *et al.*, 2001). These commercially available HS affinity columns can be used to rapidly concentrate and purify rAAV from crude cell lysate. Other groups have used the technique of high performance liquid chromatography (HPLC) to successfully purify rAAV vectors (Zolotukhin *et al.*, 1999; Gao *et al.*, 2000).

1.4.9 Titration of rAAV vectors

Reliable titration of rAAV is crucial for the planning and execution of preclinical and clinical studies and for the comparison of results between laboratories. A wide range of rAAV titres has been reported and this is due to differences between production protocols and how titres are measured and defined. The titre of rAAV can be determined by semiquantitative PCR (Miller *et al.*, 1994), quantitative real-time PCR (Sanburn and Cornetta, 1999), Southern blot (Russell *et al*, 1994) or by DNA dot blot (Flotte *et al.*, 1995) following DNase digestion of

unpackaged rAAV plasmids in vector stocks. These techniques define the number of virions containing the rAAV virion regardless of infectivity or functionality.

rAAV titres can also be determined by the replication centre assay (McLaughlin *et al.*, 1988; Yakobson *et al.*, 1987). This technique involves the infection of cells with rAAV together with wild-type AAV and adenovirus. The presence of wtAAV and adenovirus provides the necessary functions for the rAAV vector DNA to amplify within the cells. The number of cells containing amplified rAAV is determined by transferring cells onto a nylon membrane and probing with radiolabelled rAAV-specific DNA. Each dot on the autoradiograph represents one rAAV infected cell and therefore reflects the number of infectious and replication competent rAAV particles. This protocol has been modified by Salvetti *et al.* (1998) to employ a stable cell line, which expresses AAV Rep and Cap proteins. This means co-infection with wtAAV is no longer required.

rAAV vectors are also described as the number of transducing particles. This refers to the titre obtained by rAAV functional assays such as the number of GFP positive cells produced by rAAV-GFP virus from the GFP reporter gene expression.

1.4.10 Other defective viral vectors for transduction of neurons

Other than AAV, adenovirus (Ad) and herpes simplex virus (HSV) have been also used to create recombinant vectors for the transduction of neurons. Like AAV, Ad and HSV can infect post-mitotic terminally differentiated cells. Retroviruses require target cells that are undergoing cell division for genome integration and expression (Miller *et al.*, 1990) therefore neurons cannot be transduced. However,

recently non-pathogenic retroviral vectors based on lentivirus have been developed and these have the ability to infect non-dividing cells.

1.4.10.1 Adenovirus (Ad) vectors

Adenovirus is a non-enveloped virus with a genome of 36 kb, which possesses the ability to infect both dividing and non-dividing cells. The genomic region E1 is deleted in adenoviral vectors (for review see Kaplitt and Makimura, 1997). This encodes the main transactivator of Ad gene regulation, therefore deletion renders Ad-vectors replication deficient. This E1 region is complemented *in trans* to replicate the vector. This is achieved by means of the 293 cell line, which is stably transformed by the adenovirus E1 region (Hitt *et al.*, 1995). To allow the insertion of larger fragments of foreign DNA (up to 35 kb),'gutless' Ad-vectors have been generated. These contain only the ITRs, which are necessary for DNA replication, and the encapsidation signal. The other viral proteins are provided *in trans* by helper virus (Parks *et al.*, 1996; Parks and Graham, 1997). Once cells are transduced by Ad, the viral genome exists in an episomal state. Adenovirus vectors can be generated free of contaminant replication-competent virus at very high titres, and can infect both dividing and non-dividing cell. The ability to package large amounts of DNA is another of the major advantages associated with Ad-vectors, together with the fact that Ad does not integrate into the host genome therefore avoiding the risk of insertional mutagenesis. In addition, the biology of Ad virus is well known, there is a wide range of adenoviral systems available and Ad is easy to manipulate (Hitt *et al.*, 1995).

Disadvantages associated with Ad include high immunogenicity, which elicits inflammation and strong host immune responses to expressed viral antigens (Barkats *et al.*, 1998; Kozarsky and Wilson, 1993; Lawrence *et al.*, 1999), toxicity at high titres and the inability to sustain long-term expression of transgene. So far, a maximum of four months has been achieved (Geddes *et al.*, 1997; Stein *et al*, 1999).

1.4.10.2 Herpes Simplex virus-1 (HSV-1) vectors

HSV-1 is a double stranded virus with an envelope derived from the host cell and can form a lytic infection or enter a latent state in neurons. Three types of HSV-1 vectors are currently in use: amplicons, replication defective and replication competent vectors (for review see Latchman, 1999; Simonato *et al.*, 2000). The amplicons are plasmid derived vectors containing both an HSV and *E. coli* origin of replication, together with HSV packaging recognition signals. These plasmids are grown in *E. coli* and transfected into cell lines together with an HSV-1 bacterial artificial chromosome to provide helper functions (Stavropoulos and Strathdee, 1998). The primary advantage of these vectors is that they are easy to construct and multiple copies of the transgene can be delivered to neurons within each viral capsid, however the size of transgene is limited to 10 kb.

Replication defective vectors are made of mutant viruses with deletions in one or more genes essential for the lytic cycle. These can only grow in complementing cell lines, which provide the missing function. Deletion of all immediate early genes prevents virus toxicity for cells at high multiplicity of infection, which allows the vector to persist in cells for long periods (Marconi *et al.*, 1996).

Replication competent vectors are composed of attenuated viruses where genes that are not essential for replication in cultured cells are mutated or deleted. The removed genes could be important for the viral cycle in the natural host *in vivo* and could contribute to neuropathogenicity (McMenamin *et al.*, 1998). However these vectors elicit a strong cytotoxic immune response and wild-type reverents arise with a relatively high frequency during vector preparation and contaminate viral stocks with replication-competent viruses.

1.4.10.3 Lentivirus vectors

Lentivirus vectors are derived from a group of highly pathogenic retroviruses which includes the HIV viruses (for review see Lever *et al.*, 1999; Buchschacher and Wong-Staal, 2000). They have a large cloning capacity of at least 9 kb and are stably integrated into the genome of target cells. Most of the efforts so far have focused on the development of efficient vector systems based on the HIV-1 virus. In the current versions of HIV-1 based lentivirus vectors, five or six of the nine HIV-1 genes are eliminated (Zuffrey *et al.*, 1997). The viral particles are generated by transient transfection of cells with three or four different plasmids. This reduces the risk of recombination events that may lead to the generation of an infectious, replication-competent retrovirus (Zuffrey *et al.*, 1997). In addition, these HIV-1 vectors are psuedotyped with the G envelope protein of the vesicular stomatitis virus (VSV-G) which allows the vector to infect a broad range of tissues (Dull *et al.*, 1998).
The use of lentivirus vectors raises a number of important safety issues that would have to be solved before these vectors could be considered for routine use and for

clinical application. In addition, the biology and the mechanism of pathogenesis of lentiviruses are unclear, making this a difficult system to work with.

1.5 Summary

The aim of this study was to investigate transcriptional regulation of the PPT-A gene promoter in cultured DRG neurons. This was achieved by using rAAV vectors for the transduction of DRG neurons. DRG cultures are highly heterogenous and the modulation of different neuronal populations and PPT-A gene expression by individual growth factors is an extremely complex area. Therefore the cell types that are present in DRG cultures was initially established to ensure these cultures would be a suitable model system for these studies. As described in section 1.4.8, improved methods for the generation of rAAV vectors has been the focus of much research. Using certain reagents that became available over the course of this thesis, AAV vector production was first optimised to a standard suitable for the routine infection of DRG cultures. This allowed the cell types that were transduced by rAAV to be established before investigating transcriptional regulation of the PPT-A promoter. Functional analysis of the PPT-A promoter was achieved by generating a series of AAV vectors, which contained different fragments of the PPT-A promoter driving the luciferase reporter gene. The activity of these deletion constructs was examined in both cultured DRG neurons from adult and neonate rats. In addition, the effect of growth factors on the PPT-A promoter fragment (spanning nucleotides –865 to +92) was examined in adult DRG neurons. It was hoped that these experiments would lead to a greater understanding of tissue specific and inducible expression of the PPT-A

promoter in DRG neurons and generate an appropriate model system for future studies in this area.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Commonly used solutions and reagents

6 x agarose gel loading buffer	0.25% (w/v) bromophenol blue, 0.25% (w/v) Xylene cyanol, 1 mM EDTA, 30% glycerol.
TAE	1mM EDTA, 0.04 M Tris-acetate
TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
TN	20 mM Tris-HCl, 150 mM NaCl.
L-Broth	10g/l tryptone, 5g/l yeast extract, 5g/l NaCl.
L-Agar	L-broth to which 1% bacto-agar was added.
L-Agar plates	L-agar was autoclaved and left to cool to room temperature. Ampicillin was added to the agar at a final concentration of 0.1 mg/ml before pouring into plates and leaving to set at room temperature. Once set, plates were used or stored at 4 °C for upto two weeks.
Amplicillin	A stock of ampicillin was made up at 100 mg/ml and stored at -20 °C.
SOC medium	L-broth plus 10 mM glucose, 10 mM $MgSO_4$, 20 mM $MgCl_2$.

PBS	137 mM NaCl, 0.2 M $NaH_2PO4.H_2O$, 0.02 M EDTA (pH 7.4).
Collagenase/dispase	Stocks of collagenase/dispase were made up at 1.25% in PBS and stored at -20 °C
Poly-D-ornithine	Stocks were made up at 10 mg/ml in 0.15 M borate buffer and stored at –20 °C

2.1.2 Plasmids

A series of plasmids have been constructed from previous studies in this laboratory (table 2.1). These have either been used for the generation of subsequent plasmids necessary for this thesis or have been used directly.

Plasmid	Description	Reference/ Generated by
pG1-βPPTcDNA	Protein coding regions of cDNA encoding β-PPT cloned into BamHI site of pGEM1 (Promega)	MacDonald *et al.*, 1988
pSnaB1-stuffer	3277 bp HindIII-SnaBI(blunt) fragment of MHV-68 cloned with 464bp EcoRV(blunt)-SalI fragment from pATT153 (described in Sambrook *et al.*, 1989) into HindIII-SalI backbone of pGl3 basic (Promega)	Harrison *et al.*, 1999
pSub201	Infectious clone of AAV type 2 DNA	Samulski *et al.*, 1987; 1989
pGl3-865+447-LUC	1362 bp KpnI-HindIII fragment (encoding –865 to +447 of the rPPT gene relative to trasncriptional start site +1) inserted into the KpnI-HindIII sites of pGl3basic (Promega)	Fiskerstrand *et al.*, 1999; 2000
pGl3-865+92-LUC	993 bp KpnI-HindIII fragment (encoding –865 to +92 of the rPPT gene relative to the major transcriptional start site) inserted into the KpnI-HindIII sites of pGl3basic (Promega)	Fiskerstrand *et al.*, 1999; 2000
pGl3-47+92-LUC	190 bp KpnI-HindIII fragment (encoding –47 to +92 of the rPPT gene relative to the major transcriptional start site) inserted into the KpnI-HindIII sites of pGl3basic (Promega)	Fiskerstrand *et al.*, 1999; 2000
pGl3-865+447-EGFP pGl3-865+92-EGFP pGl3-47+92-EGFP	Corresponds to the luciferase plasmids described above except luciferase ORF (NcoI-XbaI) of the pGl3 plasids was replaced by an EGFP ORF (NcoI-XbaI) from pEGFP-N1 (Clonetech)	Generated by Dr. P. Harrison
pGl3-CMV-LUC	877 bp BglII-HindIII fragment of pCDNA3 (InVitrogen) carrying the CMV promoter cloned into the BglII-HindIII sites of pGl3basic (Promega)	Generated by Dr. P. Harrison
pGL3-CMV-EGFP	As pGl3-CMV-LUC except luciferase ORF replaced by EGFP	Generated by Dr. P. Harrison
pVL30	PPT promoter spanning base pairs –671 to 92 driving CAT reporter gene	From Dr. J. Quinn
pVL31	PPT promoter spanning base pairs –484 to +92 driving CAT reporter gene. This was originally described as promoter fragment –431+92.	From Dr. J. Quinn
pVL32	PPT promoter spanning base pairs –271 to +92 driving CAT reporter gene This was originally described as promoter region spanning –345 +92.	From Dr. J. Quinn
p22mut	Mutant PPT promoter spanning base pairs –865+92 driving the CAT reporter gene in which BamHI linker oligonucleotide inserted into E motif at –60 base pair.	Paterson *et al.*, 1995b
pSub201-865+447-LUC pSub201-865+92-LUC pSub201-47+92-LUC pSub201-CMV-LUC pSub201-865+92-EGFP pSub201-47+92-EGFP pSub201-CMV-EGFP	Recombinant AAV plasmid, which contains the corresponding promoter and reporter described above. Promoter and reporter DNA fragment were first inserted into pSnaB1stuffer plasmids to introduce extra DNA, then inserted into the XbaI sites of plasmid pSub201 to replace AAV genome.	Harrison *et al.*, 1999

Table 2.1: Series of plasmids provided for the purpose of this study.

2.2 Methods

2.2.1 Agarose gel electrophoresis

1% (w/v) agarose (Ultrapure agarose, GibcoBRL) was used to separate DNA fragments 7 kb to 0.5 kb and 2% agarose used to separate DNA fragments 0.5 kb to 0.1 kb. Either 100 ml or 25 ml 1 x TAE agarose gels containing 0.3 μg/ml ethidium bromide were used and placed in the appropriate gel electrophoresis apparatus (Hybaid Easi-E cast midi and mini gel electrophoresis equipment). A sufficient amount of 1 x TAE buffer was used to cover the gel. Samples were loaded in loading buffer and gels usually electrophoresed at 60 mV for as long as required to achieve separation of DNA fragments. DNA fragments were visualised using a long wave UV transilluminator.

2.2.2 Cloning and DNA techniques

2.2.2.1 Restriction enzyme digests

Typically, restriction digest reactions were carried out using 1 unit of restiction enzyme (NEB) per 1μg of DNA, 1 x reaction buffer (as specified by the manufacturer), 100 μg/ml BSA and dH_20 to the required volume. Reaction mixtures were incubated at 37 °C for 1 h. Digested DNA was visualised after agarose gel electrophoresis (section 2.2.1.). 0.2 - 0.5 μg of DNA was usually used for diagnostic digestions in a reaction volume of 20 μl. 1 - 1.5 μg DNA in a final volume of 50 μl was used in restriction enzyme digests for subcloning purposes.

DNA molecular weight markers used were 1 kb marker (GibcoBRL; 15615-616) which gave 23 fragment sizes ranging from 75 bp to 1.2 kb [75, 134, 154, 201,

220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3056, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198, 12216 bp]. λ HindIII/EcoRI DNA marker (GibcoBRL; 15612-013) was also used. Fragments ranged from 0.5 to 21 kb [125, 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 bp].

2.2.2.2 DNA ligation

Ligation reactions were carried out using 1 unit of T4 DNA ligase (Roche; 481220), 1 x ligation buffer (0.05 M Tris-Cl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg/ml BSA) and a 1:3 molar ratio of vector DNA:insert DNA (10 – 50 ng DNA), in a final volume of 10 μl. Ligation reactions were incubated at 14 °C for 16 h.

2.2.2.3 Purification of DNA from agarose gels

Digested DNA products were run on an agarose gel (section 2.2.1) for the appropriate time required to separate the DNA fragments. After visualising on the UV transilluminator, gel slices containing the DNA fragments of interest were excised using a clean scalpel blade. Gel slices were placed in a 1.5 ml eppendorf and purified using the QIAEXII gel extraction kit (Qiagen) according to manufacturers instructions. Briefly, gel slices were incubated in 800 μl Buffer QX1 and 15 μl of QIAEXII suspension and heated at 56 °C for 10 min to dissolve the agarose gel. This solution was then vortexed and centrifuged at 10000 rpm for 30 sec. The supernatant was removed and the suspension containing the DNA fragment was washed twice with Buffer PE. The DNA was then eluted from the QIAEXII suspension by resuspending it in 20-50 μl of TE. This was then centrifuged at 10000

rpmn for 30 sec and the supernatant containing the DNA fragment was transferred to a fresh eppendorf tube.

2.2.2.4 Production of blunt-ended DNA fragments

Following gel extraction, DNA fragments were treated with Klenow enzyme (NEB) to fill in the recessed 3' termini created by restriction enzyme digestion. Typically, 3 units of klenow enzyme per 100 ng of DNA was used, together with 33 μM dNTPs, 1 x klenow buffer (0.05 M Tris-Cl, pH 7.6, 0.01 M $MgCl_2$) and dH_20 to the required final volume. Reaction mixtures were incubated at 25 °C for 15 min, then klenow was heat inactivated by incubating at 75 °C for 20 min.

2.2.2.5 Transformation of competent *Escherichia coli (E.coli)*

Commercially bought INVαF One Shot™ (InVitrogen) competent cells were used for all transformations. Cells were thawed on ice and 2 μl 0.5M β-mercatoethanol added to each 50 μl aliquot of cells. 1-5 μl or 10 ng of ligation reaction was added directly to the cells, mixed by stirring gently and incubated on ice for 30 min. Cells were heat shocked for 30 sec at 42 °C and placed on ice for 2 min. 200 μl SOC medium was added to each vial and cells shaken horizontally at 37 °C for 1 h at 200 rpm. The entire transformation mix was spread on LB agar plates containing 100 μg/ml ampicillin or kanamycin. Plates were inverted and placed in a 37 °C incubator for approximately 18 h. Colonies that grew on the selective media were picked for mini-prep analysis.

2.2.2.6 Mini-prep analysis of transformed *E. coli* colonies

Single bacterial colonies were picked from freshly transformed plates, inoculated into 5 ml of LB containing the appropriate selective agent and grown for 12 – 16 h. Mini-prep DNA was prepared using QIAprep spin miniprep kit (Qiagen), according to manufacturers instructions. The Qiagen plasmid protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to a Qiagen anion-exchange resin under the appropriate low salt and pH conditions. RNA, protein and low molecular weight impurities are then removed by a medium-salt wash.

Briefly, bacterial cells were harvested by centrifuging at 5000 rpm for 2 min and resuspended in 250 µl Buffer P1. 250 µl Buffer P2 was then added, followed by the addition of 350 µl Buffer N3. The solution was then centrifuged for 10 min and the supernatant applied to a QIAprep columnn. This was centrifuged at 10,000 rpm for 30 sec and the flow-through discarded. The QIAprep spin column was washed by addition of 750 µl buffer PE, followed by centrifugation at 10,000 rpm for 30 sec and the flow-through discarded. The QIAprep column was then placed in a clean 1.5 ml microfuge tube and DNA was eluted by the addition of 50 µl Buffer EB (10 mM Tris-CL, pH 8.5) and centrifugation at 10,000 rpm for 1 min.

2.2.2.7 Large scale preparation of plasmid DNA

Single bacterial colonies were picked from freshly streaked selective plates and inoculated in 5 ml LB medium containing the appropriate selective antibiotic. Bacterial cultures were incubated at 37 °C for 8 – 12 h with vigorous shaking and then added to 100 or 250 ml LB selective medium, depending on whether the

plasmid was high or low copy number and grown at 37 °C for 16 h. DNA was then prepared using the QIAfilter maxi prep kit (Qiagen), according to instructions. The theory and process is similar to that of Qiagen miniprep DNA preparations described above (section 2.2.2.6) but on a larger scale and following elution, maxi-prep plasmid DNA is concentrated by isopropanol precipitation and purified by ethanol wash.

2.2.2.8 Polymerase chain reaction (PCR)

PCR reactions were carried out using 10 ng of template DNA, 100 ng of each oligonucleotide, 200 μM dATP, dCTP, dGTP and dTTP, 2 units of Taq polymerase (Roche; 1647679) and 1 x PCR buffer (50 mM KCl, 10 mM Tris-Hcl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin). Typically, 40 cycles of PCR (30 s 94 °C, 30 s 60 °C, 1 min 72 °C) were performed and polymerisation was continued for a further 5 min at 72 °C at the end of the last cycle.

2.2.2.9 DNA sequencing

All DNA sequencing of plasmid DNA was carried out by Mr. Ian Bennet by automated sequencing using a LI-COR DNA sequencer model 4000C (MXG-Biotech).

2.2.3 rAAV plasmid contruction: rPPT promoter constructs

2.2.3.1 PPT deletion constructs

rAAV plasmids containing varying lengths of the PPT promoter driving the luciferase reporter gene were used in this study. Plamids pSub201-865+447-LUC,

pSub201-865+92-LUC and pSub201-47+92-LUC were a gift from Dr. Patrick Harrison and the other deletion constructs pSub201-671+92-LUC, pSub201-484+92-LUC and pSub201-270+92-LUC were generated as follows. pSub201-270+92-LUC (originally described as incorporating promoter fragment –345+92, however was found to actually span –270 to +92 bp) was produced by three cloning steps. The BamHI-HindIII PPT-A 362 bp promoter fragment spanning base pairs-270+92 from plasmid pVL32 (from Dr. J. Quinn) was inserted into the BglII-HindIII sites of pGL3basic (Promega) to create plasmid pGl3-270+92-LUC. pGl3-270+92-LUC was subsequently digested with MluI-H3 and this 400 bp DNA fragment ligated to the MluI-H3 backbone of pSnaB1-60mut-LUC (section 2.2.3.2) to generate pSnaB1-270+92-LUC. Finally, pSnaB1-270+92-LUC was digested with NheI and this 4.3 kb fragment was inserted into pSub201-XbaI backbone to replace the AAV genome.

pSub201-484+92-LUC (originally described as incorporating promoter fragment –431+92, however was found to actually span –484 to +92 bp) and pSub201-671+92-LUC were generated in exactly same way as pSub201-270+92-LUC described above. The promoter fragment –484+92 was obtained from plasmid pVL31 and fragment –671+92 from pVL30 (both from Dr. J. Quinn).

2.2.3.2 PPT mutant constructs

Plasmid pSub201-60mut-LUC was generated by a number of steps. The PPT promoter fragment spanning base pairs –865+92, which contains a mutation at base pair –60 (E box sited disrupted by the insertion of oligonucleotide containing a BamHI site) was removed from plasmid p22mut (Paterson *et al.*, 1995b) by digestion with SalI and HindIII. This fragment was ligated to the XhoI-HindIII digested

backbone of pGL3basic (Promega) to generate plasmid pGL3-60mut-LUC. The MluI-HindIII 2.9 kb fragment from pGL3-60mut-LUC containing the PPT promoter and luciferase reporter gene was inserted into the MluI-XhoI digested pSnaB1stuffer backbone. This resulting plasmid, termed pSnaB1-60mut-LUC was then digested with NheI and the 4.65 kb fragment ligated to pSub201-XbaI backbone to generate pSub201-60mut-LUC.

2.2.4 rAAV plasmid construction: PPT cDNA expression constructs

2.2.4.1 Generation of plasmids pSub201-CMV-βPPT/pSub201-865+92-PPT

Plasmid pSub201-CMV-βPPT was produced in order to generate a recombinant AAV vector expressing PPT cDNA and therefore gene products SP and NKA. This required 3 separate cloning steps: The Hind III –EcoRI (blunt) 560 bp β-PPT cDNA fragment from plasmid pG1-β-PPT (gift from Dr. G. McGregor), which has been previously described (MacDonald *et al.*, 1988), was inserted into the HindIII-XbaI (blunt) backbone of plasmid pGL3-CMV-LUC. The resulting plasmid, pGL3-CMV-βPPT was digested with NotI and Eco47III and ligated to the NotI- NsiI (blunt) digested plasmid pSnaB1-stuffer. Finally, pSub201 was digested with XbaI to remove the entire AAV genome, leaving only the terminal repeats. The CMV-βPPT cassette containing stuffer DNA was isolated from pSnaB1-CMV-βPPT by digestion with NheI and inserted into the XbaI digested pSub201, generating plasmid pSub201-CMV-βPPT.

Plasmid pSub201-865+92-PPT was produced in the same way as pSub201-CMV-PPT as described above except plasmid pGl3-865+92-LUC was used in the

initial step whereby the PPT cDNA replaced the luciferase ORF. Subsequent steps were as described above for pSub201-CMV-PPT production.

2.2.4.2 Amplification of pSub201-CMV-βPPT by PCR for generation of pSub201- CMV-βPPTΔexon 3

Two separate PCR reactions were carried out to amplify the regions upstream and downstream of βPPT cDNA. Oligonucleotides (synthesised by MWG biotech) for PCR reaction (1) were 5' GGT CTA TAT AAG CAG AGC TCT C 3' (termed cdnamut1: corresponding to a region in the CMV promoter in plasmid pSub201-CMV-βPPT, upstream of PPT cDNA) and 5' C AAA GGG CTC ATG C**AT** TGC CT 3' (termed cdnamut2: corresponding to region in exon 3- base pairs 159 to 138 of PPT cDNA. The highlighted bases have been introduced to allow the incorporation of an NsiI site).

PCR reaction (2) employed oligonucleotides 5' T GGA TTA ATG **CAT** AAA CGG GAT G 3' (cdnamut3: complementary to region at exon3/intron3 boundary, base pairs 200 to 220 of PPT cDNA sequence. Highlighted bases will incorporate an NsiI site into the sequence), and oligonucleotide 5' TCC CTC TGC CAG TTA CTC CT 3' (cdnamut4: complementary to region in stuffer fragment of pSub201-CMV-βPPT, approximately 1.5 kb downstream of PPT cDNA).

Both reactions contained 10 ng of plasmid pSub201-CMV-βPPT and PCR was carried out as described in section 2.2.2.8. PCR products (1) and (2) were analysed by 2% and 1% agarose gels, respectively (section 2.2.1). Amplification of PPT fragment (1) using primers cdnamut1 and cdnamut2 resulted in a 450 bp product, and PPT fragment (2) using primers 3 and 4 produced a 1.7 kb fragment.

2.2.4.3 Cloning of PCR fragments (1) and (2) for the generation of pSub201 -CMV-PPTΔexon3 and pSub201-865+92-PPTΔexon3

PCR products PPT (1) and (2) (section 2.2.4.2) were cleaved with NsiI, agarose gel extracted (section 2.2.2.3) and ligated to the TA cloning vector pCRII (InVitrogen) (section 2.2.2.2). This involved a '3-way' ligation whereby PCR products PPT (1) and (2) ligated at the NsiI site and the A overhangs 5' and 3' in PCR products (1) and (2) respectively, allowed insertion into the TA cloning vector pCRII (InVitrogen). The resulting plasmid, pCRII-βPPTΔexon3 contained the PPT cDNA sequence which lacked exon 3. This was confirmed by restriction digestion (section 2.2.1) and sequence analysis (section 2.2.2.9).

PCRII-βPPT cDNA was then digested with HindIII-FseI and the mutated PPT cDNA fragment was inserted into HindIII-FseI digested plasmids pSub201-CMV-βPPT and pSub201-865+92-βPPT backbone to replace wild-type sequence. The presence of the mutant sequence was confirmed by restriction enzyme digest analysis (section 2.2.2.1). Plasmids were termed pSub201-CMV-βPPTΔexon3 and pSub201-865+92-βPPTΔexon3.

2.2.5 Determination of functional expression from AAV-CMV/-865+92-PPTcDNA particles

2.2.5.1 RNA extraction

Before constructs pSub201-CMV-βPPT and pSub201-865+92-βPPT were used for the generation of rAAV particles, it was necessary to ensure that they were functional and expressing PPT mRNA. BHK cells were transfected with plasmids pSub201-CMV-βPPT and pSub201-865+92-βPPT (section 2.2.6.2) and RT-PCR performed. Total RNA was isolated from cells using RNAazol B (Biogenesis). Cells

were washed once with PBS and 0.5 ml of RNAazol was added per 35 mm dish containing approximately 0.5 – 1 x 10^6 cells. The cell lysate was resuspended by passing through a pipette several times and transferring to a 1.5 ml eppendorf. 100 µl of chloroform was added per 1 ml of homogenate, samples were shaken vigorously and centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous layer was transferred into a fresh eppendorf and an equal volume of isopropanol was added. Samples were centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant removed. The RNA pellet was washed with 75% ethanol and centrifuged at 8000 rpm for 8 min at 4 °C. The pellet was dissolved in 160 µl 1mM EDTA and ethanol precipitated: 40 µl of 1 M NaCl was added together with 200 µl of 100% ethanol. Samples were incubated at –20 °C for at least 2 h. Samples were then centrifuged at 8,000 rpm for 20 min at 4 °C and RNA pellets resuspended in 20 µl 1 mM EDTA.

2.2.5.2 Reverse transcription-PCR

Single-stranded cDNA was produced using the Promega reverse transcription system, containing oligo (dT) primers which allows amplification of poly (A) mRNA only. Each reaction mix contained 5 mM $MgCl_2$, 1 x reverse transcription buffer, 1 mM each dNTP, 1 unit/µl Recombinant Rnasin Ribonuclease inhibitor, 15 units/µl AMV reverse transcriptase, 0.5 µg oligo(dT)15 primer per 1 µg of RNA and 2 µg RNA. Samples were incubated at 42 °C for 30 min, then heated at 99 °C for 5 min to inactivate the enzyme. This was followed by a 5 min incubation at 4 °C. The cDNA reaction was suitable to use directly in PCR reactions. Each PCR reaction contained 20 µl (< 1 µg of first-strand cDNA reaction), 2 mM $MgCl_2$ (with contribution from

first-strand cDNA synthesis reaction), 1 x reverse transcription buffer, 200 μM dNTPs (contributed from reverse transcription reaction) and 50 pmol of each primer PPTpr1 (5' AGAATTCAACATGAAAATCCTCGTG 3') and PPTpr2 (5' ATCTCCATCTGTGTCATGGAGAT 3'). These primers correspond to regions in exon 2 and exon 7 of the PPT cDNA, respectively. PCR reactions were performed as described in section 2.2.2.8.

2.2.5.3 Determination of SP protein expression from AAV-CMV-PPT cDNA

AAV-CMV-PPTcDNA was generated from plasmid pSub201-CMV-PPT by Genethon (section 2.2.9.7). Cultured adult DRG neurons were grown on 9 mm coverslips and infected with 0, $1x10^6$, $5x10^6$ and $1x10^7$ infectious virus particles of rAAV-CMV-PPT (section 2.2.9.1). Seven days post-infection cells were fixed with 4% paraformaldehyde at RT for 10 min and immunostained for SP expression (section 2.2.7).

2.2.6 Cell culture techniques

2.2.6.1 Cell culture growth medium

Human 293 cells (originally derived from human embryonic kidney cells transformed with adenovirus type 5 E1a and E1b genes) were a gift from Dr. P. Harrison (Cellular Physiology Research Unit, University College Cork, Cork, Ireland). HeLa human cervical carcinoma cells were obtained from Dr. K. Chapman (Dept. Molecular Medicine, MRC, University of Edinburgh, UK) and HeLaRC (derived from HeLa cells transformed with the AAV rep and cap genes) from Dr. P. Moullier (University of Nantes, France). These cells were maintained in minimum

essential medium (MEM) containing Earle's salts (GibcoBRL) supplemented with nonessential amino acids (NEAA) (GibcoBRL), 50 units penicillin and 50 μg/ml streptomycin and 10% foetal calf serum (FCS). Baby hamster kidney (BHK) cells were obtained from Miss D. Allan (Dept. Vet. Pathology, University of Edinburgh, UK) and grown in GMEM (Glasgow's modified Eagle's medium) supplemented with 10% new born calf serum (NBCS), 10% tryptose phosphate (GibcoBRL), 2 mM L-Glutamine, 50 units/ml pennicillin and 50 μg/ml streptomycin. All cell types were maintained at 37 °C in a 10%CO_2/air atmosphere.

2.2.6.2 Transfection of BHK cells

BHK cells were transfected by electroporation in normal growth medium supplemented with 0.5% NBCS using EquiBio EasyjectPlus gene pulser apparatus. Approximately 0.5-1x10^6 cells, together with 10 μg of DNA construct were electroporated at 600 V/ 1050 μF. Each electroporation was then plated on a 35 mm tissue cultures dish. Cells were harvested 48 h post-transfection.

2.2.6.3 Transfection of 293 cells

For the purpose of determining the optimal method for transfection of 293 cells, several techniques were performed. In each case, cells were seeded out at approximately 3 x 10^5 cells per well in 6 well tissue culture dishes, 24 h prior to transfection. In all protocols described below, cells were harvested 48 h post-transfection.

2.2.6.3.1 Polyethylenamine (PEI) mediated transfection

Different ratios of a 10 mM monomer solution of polyethylenamine (PEI) (Aldrich; 40,872-7) (Boussif *et al.*, 1995) and DNA were used for the transfection of 293 cells as the correct ratio is crucial for efficient transfection. 4μg and 8 μg of pSub201-CMV-LUC were diluted in 50 μl 150 mM NaCl. Increasing amounts (2, 6, 10, 15 μl) of PEI were also dissolved in 50 μl 150mM. 50 μl DNA was added to 50 μl PEI and incubated at RT for 10 min. 1.5 ml fresh medium was added to each well and the DNA/PEI mix was added dropwise to the cells. Fresh medium was added 2 h after transfection.

2.2.6.3.2 Calcium phosphate-mediated transfection

Cells were transfected by calcium phosphate (Sambrook *et al.*, 1989) as follows. Increasing amounts of plasmid pSub201-CMV-LUC (1, 2.5, 5 and 10 μg) were dissolved in a total volume of 110μl TE. 110 μl DNA was added to 125 μl HBS buffer (50 mM HEPES, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH7.12) and mixed well. 15.5 μl 2.5 M $CaCl_2$ was then slowly added to the DNA/HBS mixture whilst vortexing gently. This was incubated at RT for 30 min before adding dropwise to the cells. After 6 h of transfection, the medium was replaced with complete medium.

2.2.6.3.3. Effectene reagent- mediated transfection

Cells were transfected using Effectene reagent (Qiagen) according to the manufacturer's instructions. To determine the optimal conditions for transfection by Effectene, 0.5 μg and 1 μg pSub201-CMV-LUC were added to buffer EB to a total volume of 100 μl. 4 and 8 μl enhancer reagent was added to 0.5 and 1 μg DNA

respectively (the ratio of DNA:enhancer must be 1:8), vortexed and incubated at RT for 5 min. Effectene reagent was then added to the DNA/enhancer mix and vortexed. Different amounts of Effectene (2.5, 5, 12.5 and 25 µl) were added to both 0.5 and 1 µg DNA to establish the optimal ratio of DNA:Effectene. The DNA/Effectene mix was incubated at RT for 10 min. 1.5 ml fresh medium was added to the cells and 0.5 ml medium was added to the DNA/Effectene complex before it was added dropwise to the cells.

2.2.6.3.4 Transfection of 293 cells for rAAV production

For the production of rAAV particles, 293 cells were transfected using Effectene transfection reagent (Qiagen) (section 2.2.6.3.3) in 90mm tissue culture plates and the reagents were scaled up accordingly. Generally, 2.5 x 10^6 cells in 90 mm tissue-culture dishes were transfected with 6 µg of total DNA using 48 µl enhancer and 75 µl Effectene reagents as described above. 7 ml of fresh medium was added to the cells and 3 ml of medium added to the DNA/Effectene mix before addition to the cells.

2.2.7 Preparation of primary neuronal cultures

2.2.7.1 Adult dorsal root ganglion neurons

Dorsal root ganglia (DRG) with attached roots were dissected from Wistar Albino rats (4-6 weeks) and collected in PBS. Ganglia were freed of connective tissue and nerve roots and each ganglia cut into approximately 4 pieces. Ganglia were treated with 0.125% collagenase /dispase (Roche; 269-638) for 2 h at 37 °C. Dissociation of neurons was obtaining by resuspending ganglia approximately 15

times using a 1 ml pipette and allowing the undissociated ganglia to fall to the bottom of the tube. The supernatant containing dissociated ganglia was transferred to fresh tube. This process was repeated as many times as necessary. Neurons were centrifuged for 1000 rpm 2 min, resuspended in F-14 medium (GRH Biosciences) containing 10% FCS and plated onto poly-D-ornithine (0.5 mg/ml in 0.15 M borate buffer (Sigma; P8638) coated 35 mm tissue culture dish. After 15 hours, the non-neuronal cells are firmly attached to the dish, while the neurons only weakly adhere. The following day, culture medium was carefully removed and neurons dislodged from the plate and collected in a 15 ml conical tube. Cells were centrifuged at 1000 rpm for 2 min and the cell pellet resuspended in F-14 medium containing 10% FCS and 100 ng/ml NGF (7S, Promega). Cells were plated on poly-D-ornithine and laminin (1 mg/ml Roche; 23017-015) coated 9 mm coverslips. 2 days after culturing cells were maintained in F-14 medium containing 2% FCS and medium was changed every 3 – 4 days.

2.2.7.2 Neonate dorsal root ganglion neurons

Dorsal root ganglia were removed from 1 day old pups (Wistar Albino) and collected in PBS. Ganglia were freed of roots and treated with 0.125% collagenase/dispase for 45 min at 37 °C. Ganglia were washed 3 times with PBS and then 3 times with medium solution 1 (Dulbecco's Modified Eagles Medium containing 200 mM L-glutamine, 1% penicillin/streptomycin and 10% FCS). DRG neurons were triturated by passing the ganglia through a 21G syringe needle 8 times and then passing through a 23G needle until cells are fully dispersed. The cell suspension was then passed through a 40 μm cell strainer (Falcon) and centrifuged

1000 rpm 2 min. The cell pellet was resuspended in medium containing 100 ng/ml NGF and 2.5 μM Cytosine Arabinoside (Sigma; C1768) and plated onto poly-L-lysine and laminin coated coverslips. 48 hours after plating, cells were replaced with medium solution 2 (DMEM containing L-glutamine, penicillin/streptomycin, 1% FCS and 1 x N-2 supplement (Gibco; 17502-048). Cytosine arabinoside is a selective inhibitor of DNA synthesis and N-2 supplement allows the maintenance of rat primary neurons in mass culture.

2.2.8 Immunostaining of cultured DRG neurons

Cells were fixed by incubating with 4% paraformaldehyde for 15 min at RT. Cells were washed 5 times in PBS and then incubated for 10 min at RT in PBS containing 0.2% triton-X. Cells were again washed several times with PBS and then incubated with block solution (PBS containing 20% normal goats serum) for 30 min at RT. Cells were then incubated at 4 °C overnight in primary antibody.

Rabbit anti-SP antibody (Penninsula laboratories) was used at a working dilution of 1:20,000 in block solution and detected using a 1:200 working dilution of goat anti-rabbit IgG (10 μg/ml; Vector laboratories). Mouse anti-neurofilament antibodies (monoclonal anti-neurofilament 200, clone N52, Sigma) was used at a working dilution of 1:400 of antibody solutions provided (no details of SP and NF antibody concentrations were provided) and was detected by biotinylated goat anti-mouse IgG (used at 1:200 working dilution, 5 μg/ml, Vector). For IB-4 analysis of DRG neurons cultures were incubated with biotinylated IB-4 (Sigma) at a working dilution of 1:500 (0.4 μg/ml). In most cases, staining was visualised using Vector Alkaline Phosphatase Substrate kit I (Vector laboratories SK-5100).

After overnight incubations with primary antibodies, cultures were washed with PBS 5 times and incubated with secondary biotinylated antibody for 1 h at RT. Cells were then washed several times with PBS and incubated with Vectastain ABC-AP (Vector laboratories) complex for 30 min. The ABC-AP complex was made according to manufacturer's instructions. These ABC-AP kits contain a special form of Avidin DH and biotinylated alkaline phosphatase H. To make up the ABC complex, 1 drop of reagent A and 1 drop of reagent B were added to 5 ml buffer (10 mM sodium phosphate, pH7.5, 0.9% PBS). This was mixed well and incubated at RT for 30 min before adding to the cells. Cells were again washed several times with PBS and the colour reaction developed as follow.

The alkaline phosphatase substrate working solution was prepared according to manufacturers instructions: To 5 ml 100mM tris-HCl, pH 8.2-8.5, 2 drops of reagent 1 was added and mixed well. Then 2 drops of reagent 2 was added and mixed well and 2 drops of reagent 3 was added and mixed well. This was added immediately to the cells. The colour was developed for approximately 20 mins before it was stopped by the addition of tap water.

Cells were then counterstained for 5 seconds in haematoxylin, washed in Scotts water and then in tap water and mounted using aqueous mount (Aquamount improved, BDH; 362262H). In control experiments the primary antibody was omitted.

2.2.9 Virus techniques

2.2.9.1 Adenovirus production

Both wild-type adenovirus type 2 and mutant adenovirus type 5 varient ΔE1A/E1B were produced by the following method. 60 – 80% confluent 293 cells in 175 cm^2 tissue-culture flask were infected with virus at a multiplicity of infection (moi) of 1 in growth medium supplemented with 2% FCS and incubated at 37 °C for 1 h to allow viral adherence. Normal growth medium (containing 10% FCS) was then added and infected cells were incubated at 37 °C for 4 – 7 days until cytopathic effects were observed. Cells were then harvested and centrifuged at 1000 rpm for 5 min. The supernatant was removed and cell pellet resuspended in TE buffer. Cells were freeze-thawed three times and spun at 1000 rpm for 4 min to remove cell debris. The supernatant containing adenovirus was transferred to a fresh eppendorf and stored in 50 µl aliquots at – 70 °C.

2.2.9.2 Titration of adenovirus

Both wild-type adenovirus type 2 and mutant adenovirus type 5 titres were established by end point dilution. Cells were seeded out in 96 well plates at approximately 30% confluency. The following day, adenovirus stock was diluted in medium from 10^{-6} through to 10^{-11}. 100 µl of each dilution was added to 10 wells and incubated for 16 h before changing the medium. Medium was then changed every 3 days and the number of viral plaques at each dilution counted 6 to 8 days post-infection. The method of Reed and Meunch was used to calculate $TCID_{50}$/ml and virus titre pfu/ml (Revah *et al.*, 1996) (section 2.2.9.3).

2.2.9.3 Reed and Muench method to calculate $TCID_{50}$/ml

The 50% infectious dose ($TCID_{50}$ or tissue culture 50% infectious dose) is calculated using mathematical anaylsis of the data. The Reed and Muench method was used to determine adenovirus titre after counting plaques generated by various dilutions of adenovirus stocks as described above (section 2.2.9.2). The resulting adenovirus titres are expressed as plaque forming units per ml (pfu/ml).

$$I = \frac{(\%\text{ of wells infected at dilution above 50\% - 50\%})}{(\%\text{ of wells infected at dilution above 50\% - \% wells infected at dilution below})}$$

$$50\%\text{ endpoint titre} = 10^{\log \text{total dilution above 50\% - (I x log h)}}$$

Where I = Interpolated value of the 50% endpoint (or proportionate distance)

h = dilution factor

Example of the method of Reed and Meunch to calculate titre:

If a titration gave 9/10 positive wells (90%) at the 10^{-8} dilution and above, 3/10 (30%) at the 10^{-9} dilution and 0/10 at the 10^{-10} dilution then:

$$I = \frac{(90\% - 50\%)}{(90\%-30\%)} = \frac{40}{60} = 0.67$$

$$50\%\text{ endpoint titre} = 10^{-8 - (0.67 \times 1)}$$

$$50\%\text{ endoint titre} = 10^{8.67}$$

This represents the number of infectious doses per unit volume therefore if only 100 µl of virus was added to each well, this must be multiplied by 10 to give a final value of $10^{9.67}$ $TCID_{50}$/ml.

1 TCID is approximately equal to 0.7 plaque forming units (pfu) and therefore can define titre as pfu/ml.

2.2.9.4 Preparation of rAAV

In these studies rAAV was initially generated using AAV helper plasmid (pXX2) and AAV plasmid (pSub201) together with adenovirus mutant to provide helper functions. Later plasmid pXX6-80 (Xiao *et al.*,1998) was used in place of adenovirus.

2.2.9.4.1 Generation of rAAV using mutant adenovirus as helper

90 mm tissue culture dishes containing 50 – 80% confluent HEK293 cells were transfected with 4 µg AAV plasmid and 2 µg pXX2 using Effectene transfection reagent (section 2.2.6.3.4). 6 h post-transfection, cells were infected with adenovirus mutant in medium containing 2% FCS (section 2.2.9.1) at approximate moi of 10. 1 h after adenovirus infection, medium was replaced with complete medium and cells were incubated for approximately 48-72 hours. Cells were harvested when a cytopathic effect was apparent (when all cells looked rounded and infected but still attached to plates). Cells were centrifuged for 4 min at 2000 rpm and cell pellet resuspended in 1 ml TN. Cells were then subjected to 3 rounds of freeze-thawing and then centrifuged at 2000 rpm for 4 min. The supernatant was collected in a fresh tube and heated at 56 °C for 45 min to inactivate adenovirus. This

was then centrifuged at 2000 rpm for 2 min and the supernatant collected in fresh tube. rAAV preps were stored in 50µl aliquots at –70 °C.

2.2.9.4.2 Generation of rAAV using pXX6-80 as helper

293 cells, approximately 80% confluent were transfected by Effectene transfection reagent (section 2.2.6.3.4) with 1 µg AAV plasmid (pSub201), 2 µg pXX2 and 3 µg pXX6-80 (Xiao *et al.*, 1998). 72 h post-transfection, cells were harvested by resuspending at 2000 rpm for 4 min and cell pellet resuspended in 1 ml TN (100 mM Tris-HCl, pH 7.5, 150 mM NaCl). Cells were then freeze-thawed three times and centrifuged at 2000 rpm for 4 min. rAAV preparations were aliquoted and stored at -70 °C.

2.2.9.5 Optimisation of rAAV production

To determine the optimal reagents for rAAV production, rAAV titres were compared when AAV was generated using AAV helper plasmid pAAV/Ad compared with pXX2. Production of rAAV was also compared when adenovirus and plasmid pXX6-80 provided helper functions and when different ratios of these plasmids were used. In all experiments, pSub201-CMV-LUC was used to generate rAAV-CMV-LUC. Approximatley 2.5 x 10^5 293 cells per well were seeded in 6 well plates 20 h prior to transfection and transfected using PEI (section 2.2.6.3.1) and harvested 48 h post-transfection.

In the first set of experiments, production of rAAV-CMV-LUC was investigated when pAAV/Ad and mutant adenovirus are used. The amount of pSub201-CMV-LUV and pAAV/Ad used was varied. Ratios of 1:3, 3:1 and 1:1 µg

of pSub201-CMV-LUC:pAAV/Ad were compared. A similar experiment was carried out in which pXX2 was used as the AAV helper plasmid. Cells were transfected using PEI at the appropriate DNA:PEI ratio as described in section 2.2.6.3.1. Briefly, 4 μg total DNA was diluted in 50 μl 150 mM NaCl and 2 μl PEI was diluted in 50 μl 150 mM NaCl. These were mixed together and incubated at RT for 10 min before adding to the cells and fresh medium was added 2 h post-transfection. 6 h post-transfection, cells were infected with mutant adenovirus as follows. The medium was removed and replaced with 500 μl growth medium containing 2% FCS and adenovirus at a moi of 10. This was incubated for 1 h before 2 ml complete medium (containing 10% FCS) was added to the cells.

A third experiment investigated AAV production when plasmid pXX6-80 is employed. Varying ratios of plasmids were compared: 0.8: 0.8: 2.4, 0.8:1.6:2.4, 0.8:0.8:4.8 and 0.8:1.6:4.8 μg of pSub201-CMV-LUC: pXX2: pXX6-80 respectively were used for transfection of 293 cells by PEI as described above. Cells were harvested 72 h post-transfection by centrifuging at 2000 rpm for 4 min. The cell pellet was resuspended in 250 μl TN and cells were subjected to three rounds of freeze-thawing and the supernatants processed as described in section 2.2.8.3. rAAV preparations were then titred by the modified replication centre assay (section 2.2.9.6).

2.2.9.6 Titration of rAAV particles

rAAV titres were determined by the modified replication centre assay (Salvetti *et al.*, 1998). HeLaRC cells were seeded at approximately 3 x 10^6 cells per 24 well plate. The following day, cells were infected with varying concentrations of

the rAAV samples, diluted 10^{-2}, 10^{-3},10^{-4}, 10^{-5} and 10^{-6} in 100 µl Optimem (GibCo), together with wild-type adenovirus type 2 at an moi of 20 in 100 µl Optimem. Cells were incubated 37 °C for 2 h then 1 ml complete medium was added to the infected cells. Cells were harvested 30 h post-infection and cell pellet resuspended in 4 ml versene. Cells were then applied to nylon membrane filters (Schleicher and Schuell, 1- 414112) using dot-blot apparatus and left to air dry. Membrane filters were washed once in 0.5M NaOH/1.5M NaCl for 5 min and twice in 1 MTris pH7.2 x SSC 5 min and left to air-dry overnight. The following day filters were baked at 80 °C for 30 min and washed in pre-hybridisation buffer (50% formaldehyde, 5 x SSC, 5 x denhardts, 100 mM NaH_2PO_4, 17 mM SDS) for 2 h.

To probe for AAV particles, DNA fragments containing the luciferase ORF was ^{32}P labelled using 'Ready-to-go'^{TM} DNA labelling beads (Amersham pharmacia biotech) according to manufacturers instructions. Approximately 25-50 ng DNA was made up to a final volume of 45 µl in dH_20 and denatured by heating for 3 min at 99 °C. It was then placed immediately on ice for 2 min and centrifuged briefly. The denatured DNA was then added to the DNA labelling beads (contain labelling beads, buffer, dATP, dGTP, dTTP, klenow fragments of DNA polymerase, random nucleotides) and resuspended. 50 µCi [α-^{32}P] dCTP was added to the DNA/bead mix and incubated at 37 °C 30 min. The labelled DNA was then isolated from free probe by using Probe-Quant^{TM} G-50 microcolumns (Amersham pharmacia biotech) according to manufacturers instructions. Briefly, ^{32}P-DNA was added to the column and centrifuged at 2000 rpm for 1 min. The flow-through was then boiled for 2 min and placed on ice. 100 µl prehybridisation buffer was added to the ^{32}P-labelled DNA and this was incubated with the filters overnight at 37 °C.

2.2.9.7 Generation of rAAV vectors by Genethon

Genethon is a charitable organisation that provides researchers with batches of vectors, reagents and technical assistance for those working in the field of virus vectors and gene therapy. Plasmids pSub201-CMV-PPT, pSub201-CMV-PPTΔexon3, pSub201-CMV-GFP and pSub201-PPT-GFP were send to Genethon for generation into rAAV particles. rAAV vectors were generated using a three plamsid protocol to avoid use of adenovirus to provide helper functions. This resulted in the production of clean, high titre rAAV prepartions that are free from contaminating adenovirus. Following rAAV production, rAAV stocks are purified by two CsCl gradients and titred.

2.2.10 Transduction of DRG cultures by rAAV

2.2.10.1 Infection of DRG cultures by rAAV

DRG cultures were grown on 9 mm glass coverslips. Coverslips containing cells for infection were transferred into a well of a 48 well culture dish. The rAAV was then added to the cells in a total volume of 150 µl F-14 containing 10% FCS. The following day the medium was removed and 250 µl fresh medium was added.

2.2.10.2 Growth factor induction

For growth factor experiments, the medium of DRG cultures was replaced 24 to 48 h after neurons were plated on coverslips, with F14 containing 10% FCS only. Cells were incubated for at least 24 h before infecting with the rAAV (section 2.2.10.1). Upon infection, rAAV was added in F14 medium containing 10% FCS + /- growth factor. Cells were maintained in medium containing the growth factor for the

duration of infection time. Growth factors used were 100 ng/ml 7s NGF (mouse recombinant, Promega), 25 ng/ml LIF (mouse recombinant, Sigma, L5158), 25 ng/ml BDNF (human recombinant, Sigma B3795), GDNF (rat recombinant, Sigma G1401), 50 ng/ml CNTF (rat recombinant, Sigma C3960) and IL-6 (mouse recombinant, Sigma I9646).

2.2.10.3 Detection of luciferase expressing rAAV vectors: luciferase assays

Luciferase activity was determined using the Promega Luciferase assay kit according to manufacturers instructions. For DRG exepriments, cultures that were infected with rAAV as described in section 2.2.10.1 were harvested and assayed as follows. Cultures in 48 well plates were washed gently with PBS and 70 µl of reporter lysis buffer was added to the cells. Cells were incubated in lysis buffer for 15 min and the lysate transferred to a fresh eppendorf. Samples were freeze-thawed, vortexed briefly, centrifuged at 10,000 rpm for 1 min and the supernatant transferred to a fresh eppendorf. 40 µl of lysate was mixed with 200 µl of luciferase assay buffer and luciferase activity measured using a luminoskan RT luminometer.

For cell culture that had been transfected with pSub201-CMV-LUC (section 2.2.6.3), after washing with PBS, 250 µl of 1 x lysis buffer was added to each well of the 6 well tissue culture dishes. The cell lysate was treated as described above for DRG cell lysates. To assay for luciferase activity, 1 µl of lysate was first diluted in 50 µl of lysis buffer and then 20 µl of diluted lysate was mixed with 100 µl of luciferase assay buffer.

2.2.10.4 Detection of GFP expressing rAAV vectors

Following infection of DRG cultures with AAV-PPT-GFP or AAV-CMV-GFP, GFP expression could be detected by fluorescent microscopy. In most cases, cells were fixed and subject to ABC immunostaining. Cells were fixed, washed and blocked as in section 2.2.7 and then incubated overnight with 0.1 μg/ml mouse anti-GFP (Molecular probes). Cells were washed and incubated for 2 h with goat anti-mouse biotinylated secondary antibody and stained using ABC-AP and Vector red kit as described in section 2.2.7. In some experiments GFP antibodies were visualised by Vector SG substrate kit and Elite ABC kit. The Elite ABC kit contains Avidin DH and biotinylated horseradish peroxidase H reagents, which form complexes for immunoperoxidase staining. Elite ABC complex was made according to manufacturer's instructions: 2 drops of reagent A was added to 5 ml buffer and mixed well. 2 drops of reagent B was then added and mixed well. To prepare vector SG colour reaction, 3 drops of Chromogen was added to 5 ml PBS and mixed well. This was followed by the addition of 3 drops of hydrogen peroxidase solution. Colour reactions were developed for approximately 5 min and stopped by the addition of tap water.

CHAPTER 3: TRANSDUCTION OF DRG NEURONS BY AAV VECTORS

3.1 Introduction

The aim of this study was to optimise the production of recombinant AAV (rAAV) vectors to a standard suitable for the routine transduction of cultured DRG neurons to investigate PPT-A promoter regulation in sensory neurons. Previous work in this laboratory had employed rAAV technology for transfection of DRG neurons to investigate stimulus inducibility of the PPT promoter (Harrison *et al.*, 1999). However the techniques available for generating rAAV vectors have since improved and many questions regarding rAAV infection of DRG neurons remain to be answered. To address these issues, the protocol for generating rAAV vectors was optimised to increase rAAV vector titres and eliminate the need for adenovirus helper function. Subsequently, the conditions necessary for rAAV infection of DRG cultures and the DRG cell populations that support reporter gene expression from rAAV vectors were also addressed.

3.2 Optimisation of rAAV production

Over the course of this thesis the technology for generating rAAV has rapidly improved, allowing high titre preparations that are pure and free of adenovirus contamination (section 1.4.8.1). The original protocol (figure 3.1) for the production of rAAV particles involves the transient transfection of 293 cells with a rAAV packaging substrate and helper plasmid. Subsequently superinfection with mutant adenovirus provides the essential proteins for AAV replication and packaging (Samulski *et al.*, 1989). The AAV packaging substrate retains only the 145 bp inverted terminal repeats (ITR) flanking the gene of choice and the AAV structural

proteins are provided in *trans* by cotransfection of the AAV packaging substrate with a helper plasmid containing only the AAV *rep* and *cap* genes. In the presence of adenovirus infection, expression of the AAV *rep* and *cap* genes occur. This allows replication of rAAV sequences and single-stranded DNA molecules to be encapsulated into AAV capsid structures. This method generates both rAAV particles and adenovirus therefore rAAV is subsequently isolated by inactivation of adenovirus (section 1.4.7; figure 3.1).

This method has several limitations as it can be technically difficult and inefficient, it can be difficult to achieve high titres and there is the possibility that residual adenovirus may contaminate stocks. Therefore focus has been on improving AAV production (section 1.4.8). One area has been the development of packaging cell lines, which express the AAV rep and cap genes, to allow large scale production (Holscher *et al.*, 1994, Tamayose *et al.*, 1996, Inque and Russell, 1998). Other groups have generated improved AAV helper plasmids, in which the AAV genes essential for DNA replication are down-regulated, to increase rAAV titres (Xiao *et al.*, 1998). To eliminate the possibility of adenovirus contamination, adenovirus-AAV helper plasmids have been generated (Collaco *et al.*, 1999; Matsushita *et al.*, 1998; Grimm *et al.*, 1998) which carry both essential adenovirus and AAV helper function. In addition, an adenovirus plasmid has been generated which is incapable of producing infectious adenovirus particles but can provide all the necessary helper functions for rAAV replication (Xiao *et al.*, 1998; section 1.4.8.1).

As many of these reagents have become available over the course of this study we were able to take advantage of these to improve and optimise the conditions for generating rAAV in this laboratory. The work presented here employs the

adenovirus helper plasmid described by Xiao *et al.* (1998) in place of mutant adenovirus to improve rAAV titres and also avoid adenovirus contamination.

Initially in this laboratory the original protocol employing mutant adenovirus was used for the generation of AAV particles (figure 3.1). This therefore involved the transfection of 293 cells by electroporation with the helper plasmid pAAV/Ad together with pSub201 plasmids (AAV packaging substrate), followed by infection with mutant adenovirus (Samulski *et al.*, 1989). However, transfection of 293 cells by electroporation generally caused high amounts of cell death and was thought to be quite an inefficient method for transfection, resulting in low titre rAAV preparations. The first step in this optimisation process was therefore to improve the transfection efficiency of 293 cells. Consequently, production of rAAV using the standard method described was compared to protocols that employed plasmids pXX2 and pXX6-80 (Xiao *et al.*, 1998) instead of AAV helper plasmid pAAV/Ad and adenovirus respectively. Plasmid pXX2 is an improved AAV helper plasmid and plasmid pXX6-80 carries the genes essential for adenovirus helper functions. Over the course of this study these two important reagents came available and were shown by Xiao *et al.* (1998) to be more effective in generating high titre rAAV that is free of contaminating adenovirus.

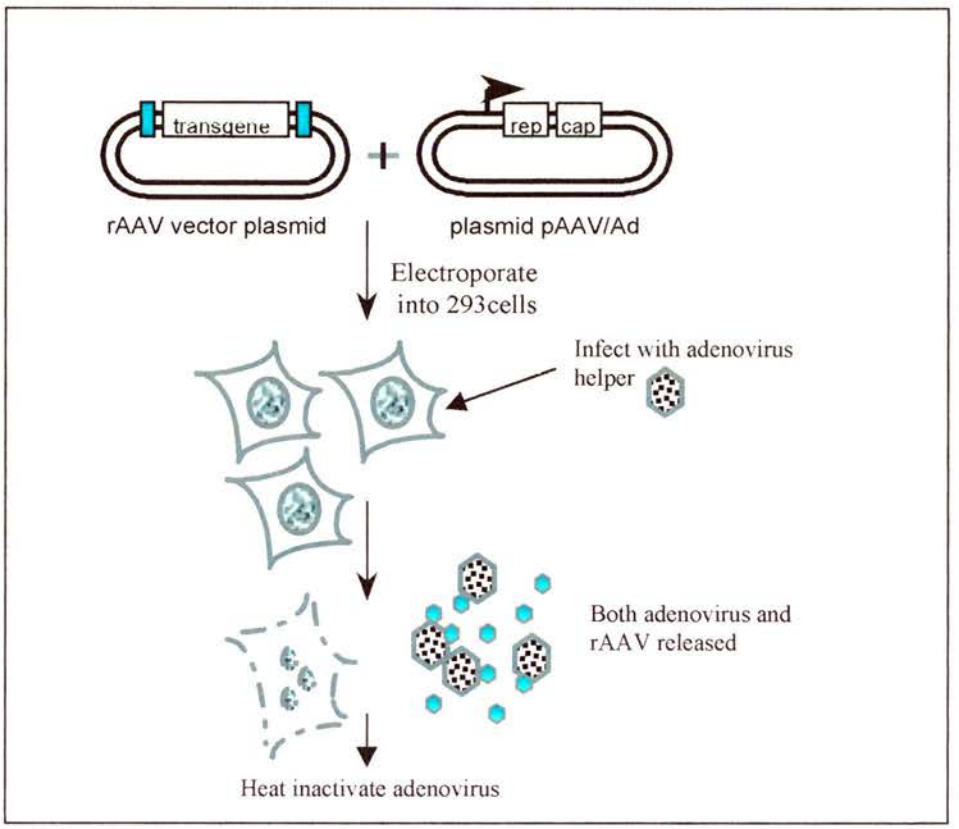

Figure 3.1: Standard protocol for the production of rAAV. 293 cells are transfected with rAAV helper plasmid and the AAV plasmid and are then infected with adenovirus. The AAV helper plasmid pAAV/Ad provides the AAV genome *in trans* resulting in adenovirus and rAAV particles production. Both viruses are released and adenovirus is then inactivated by heating to 56 °C.

3.2.1 Transfection of 293 cells

To determine the most effective method for transfection of 293 cells, cells were transfected with plasmid pSub201-CMV-LUC by calcium phosphate, polyethylenimine and Effectene reagent (Qiagen) (section 2.2.6.3). In all experiments, 2.5 x 10^6 293 cells were seeded out approximately 20 h prior to transfection in 6 well tissue culture dishes and were approximately 70 – 80% confluent on the day of use. Cells were harvested 48 hours post-transfection and lysed extracts assayed for luciferase activity (section 2.2.10.3).

3.2.1.1 Effectene (Qiagen) reagent-mediated transfection

Effectene transfection reagent is a non-liposomal highly branched lipid molecule that works in conjunction with a DNA-condensing enhancer. The first step in Effectene mediated transfection is incubation of DNA with the enhancer reagent to condense the DNA. Effectene reagent is then added to this complex and the DNA-Effectene complex is added directly to the cells. The DNA:Effectene complex requires a slight positive charge in order to bind efficiently to the negatively charged cell surface molecules and successfully transfect cells. It was therefore necessary to optimise the amount of DNA and Effectene reagent used since the overall charge of the Effectene DNA complex is determined by the ratio of Effectene to DNA-enhancer mix (section 2.2.6.3.3). 0.5 μg DNA was used together with 2.5, 5, 12.5 and 25 μl Effectene to give DNA (μg):Effectene (μl) ratios of 1:5, 1:10, 1:25 and 1:50 respectively. 1 μg DNA was also used together with 2.5, 5, 12.5 and 25 μl Effectene to give ratios of 1:2.5, 1: 5, 1:12.5 and 1:25 respectively. It was found that

1 μg DNA used in combination with 25 μl Effectene reagent reproducibly resulted in the highest transfection effciencies (table 3.1).

3.2.1.2 Polyethylenimine-mediated transfection

Polyethylenimine (PEI) molecules are highly positively charged and when added to DNA, form microprecipitates that are absorbed by the cells by endocytosis (Boussif *et al.*, 1995). To investigate transfection efficiency of 293 cells by PEI, different ratios of DNA:PEI were also tested (section 2.2.6.1). Both 4 μg and 8 μg DNA was each used in combination with 2, 6, 10 and 15 μl 10 mM PEI. 4 μg of DNA together with 2 μl of PEI (DNA:PEI ratio of 1:05) resulted in the highest luciferase values (table 3.2). Increased ratios of DNA:PEI was observed to be toxic to the cells and resulted in reduced transfection efficiencies. This suggested that DNA:PEI ratios are critical for achieving efficient transfection.

3.2.1.3 Calcium phosphate-mediated transfection

Calcium phosphate-mediated transfection (section 2.2.6.3.2) is the most commonly used method for transfection of 293 cells for the production of AAV, particularly when large scale preparations are generated as this technique is inexpensive and relatively efficient. An exact pH is essential for efficient transfection and pH 7.12 was found to be critical. The amount of DNA that was optimal for the formation of calcium phosphate-DNA coprecipitates at pH 7.12 was determined. Increasing amounts of DNA in a total volume of 110 μl were added together with 125 μl 2 x HBS pH 7.12. 15.5 μl 2.5 mM calcium chloride was then added to the DNA/HBS mix, which was then incubated with the cells in 6 well culture dishes. 1,

2.5, 5 and 10 μg DNA was compared and it was found that 1 μg DNA achieved the highest transfection efficiency (table 3.3).

3.2.1.4 Comparison of transfection procedures

PEI-mediated transfection resulted in the highest luciferase values when a DNA:PEI ratio of 1:0.5 was used (tables 3.1 to 3.3). Increasing amounts of PEI were observed to be highly toxic to the cells. Table 3.2 shows there is a 6 fold reduction in transfection efficiency when a DNA:PEI ratio of 1:1.5 was used, indicating that the DNA:PEI is crucial for effective transfection.

Effectene reagent-mediated transfection was shown to provide high transfection efficiencies as measured by luciferase activity and was the most effective method of transfection (table 3.1). Unlike PEI mediated transfection, a large variation in transfection efficiency was not observed between the different DNA:transfection reagent ratios when Effectene reagent was employed. In addition, toxicity to the 293 cells was not apparent and high levels of transfection are achievable despite using much lower quantities of DNA. To achieve these high transfection efficiencies only 1 μg DNA was required, whereas to achieve equivalent luciferase values by PEI mediated transfection, it was necessary to use 4 μg DNA. This was an important consideration as difficulty was encountered in achieving high yields of adenovirus helper plasmid pXX6-80 from plasmid preparations. Subsequently, Effectene transfection reagent at a DNA:Effectene ratio of 1:25 has been the method of choice for transfection of 293 cells for the production of rAAV.

Amount of DNA (μg)	Amount of Effectene (μl)	Ratio DNA: Effectene	Luciferase reading	Protein conc. (μg/ml)	Luciferase activity/μg protein
0.5	2.5	1:5	1406	270	260
	5	1:10	3761	275	684
	12.5	1:25	2445	260	470
	25	1:50	3448	505	341
1	2.5	1:2.5	1157	270	214
	5	1:5	4438	340	653
	12.5	1:12.5	8182	270	1515
	25	1:25	10213	300	1702

Table 3.1: Luciferase values of cell extract of 293 cells transfected with pSub201-CMV-LUC by Effectene transfection reagent. To assay, cell extract was diluted 1:50, then 20 μl cell extract was added to 100 μl luciferase assay buffer.

Amount of DNA (μg)	Amount of PEI (μl)	Ratio DNA:PEI	Luciferase reading	Protein conc. (μg/ml)	Luciferase activity/μg protein
4	2	1:0.5	12454	330	1887
	6	1:1.5	1909	250	382
	10	1:2.5	1134	280	203
	15	1.3.75	75	120	31
8	2	1:0.25	8539	270	1581
	6	1:0.75	1294	250	259
	10	1:1.25	789	150	263
	15	1.1.9	590	130	227

Table 3.2: Luciferase values of cell extract of 293 cells transfected with pSub201-CMV-LUC by polyethylenimine. To assay, cell extract was diluted 1:50, then 20 μl cell extract was added to 100 μl luciferase assay buffer.

Amount of DNA (μg)	Luciferase reading	Protein conc. (μg/ml)	Luciferase activity/μg protein
1	1532	270	284
2.5	149	210	35
5	390	300	65
10	30	250	6

Table 3.3: Luciferase values of cell extract of 293 cells transfected with pSub201-CMV-LUC by calcium phosphate method . To assay, cell extract was diluted 1:50, then 20 μl cell extract was added to 100 μl luciferase assay buffer.

3.2.2. Production of rAAV vectors

To address the issue of improving rAAV yield and producing stocks, which are completely free of helper adenovirus, plasmids pXX2 and pXX6-80 generated by Xiao *et al.* (1998) were utilised (figure 3.2). pXX2 is an AAV helper plasmid which was designed to specifically increase rAAV titres. In comparison to the original packaging plasmid pAAV/Ad, pXX2 has attenuated synthesis of AAV Rep78/68 proteins and an additional p5 promoter downstream of the AAV Cap open reading frame. AAV Rep 78/68 is essential for DNA replication however unregulated overexpression is known to inhibit rAAV production, therefore reduction of Rep78/68 expression by attenuated translation initiation has resulted in much higher AAV yields (Li *et al.*, 1997). Deletion of the p5 promoter results in down-regulation of promoters p19 and p40 (Pereira *et al.*, 1997), which allow transcription of genes coding for Rep52/40 and the capsid proteins. It was therefore thought that the additional p5 promoter would increase the expression of Rep 52/40 and the capsid proteins. Rep 52/40 is important for AAV single-stranded formation, which is part of the packaging process (Chejanovsky and Carter, 1989), and the capsid proteins are required for virus assembly. The collective effects of these helper plasmid changes were shown to result in higher rAAV yields (Xiao *et al.*, 1998).

A number of adenovirus genes, including E1a, E1b, E2a, E4 and VA genes are necessary for helper function (Berns, 1996). pXX6-80 contains only the E2a, E4 and VA genes and is capable of propagating rAAV in the presence of AAV *rep* and *cap* genes in 293 cells. These cells provide the missing adenovirus E1a and E1b genes, which are essential for replication. pXX6-80 is defective for adenovirus replication and the ability to produce adenovirus structural proteins, however as it

can still provide helper function, rAAV vectors will be free of adenovirus contamination.

To optimise the conditions for generating rAAV using these reagents, rAAV stocks were made using the packaging plasmids pAAV/Ad or pXX2 together with adenovirus as helper. The titres of these rAAV preparations were then compared to rAAV stocks that were generated when pXX6-80 was employed instead of adenovirus. AAV packaging substrate pSub201-CMV-LUC was used for producing rAAV-CMV-LUC stocks in all experiments and different ratios of pSub201-CMV-LUC: AAV helper plasmid was also compared. Figure 3.2 shows the plasmids used for this study and figure 3.3 summarises the conditions of each experiment.

For these initial optimisation experiments PEI was utilised as it is inexpensive and achieves efficient transfection of 293 cells. Cells were seeded out 20 h prior to transfection in 6 well tissue culture dishes and were approximately 80% confluent on day of use. Cells were transfected with plasmids by PEI-mediated transfection as described in section 3.2.1.2. (see section 2.2.9.5 for a more detailed description). When adenovirus was used as helper, cells were infected 2 h post transfection and harvested approximately 48 h following infection, when cytopathic effects were apparent. However when pXX6-80 was co-transfected with pXX2 and pSub201-CMV-LUC, cells were harvested 72 h post-transfection. Harvested cells were lysed by three cycles of freeze thawing, cell debris was removed and adenovirus was heat inactivated when present (section 2.2.9.5). HeLaRC cells were then infected with varying concentrations of the rAAV preparations and the titres determined by the modified replication centre assay (section 2.2.9.6).

The highest titres of rAAV were obtained when pXX2 and pXX6-80 were used to prepare stocks (table 3.5) compared to those rAAV preparations generated when pAAV/Ad helper or pXX2 was utilised together with adenovirus (table 3.4). Specifically, the greatest titres were achieved when plasmids pSub201-CMV-LUC, pXX2 and pXX6-80 were transfected at a ratio of 1:2:3.

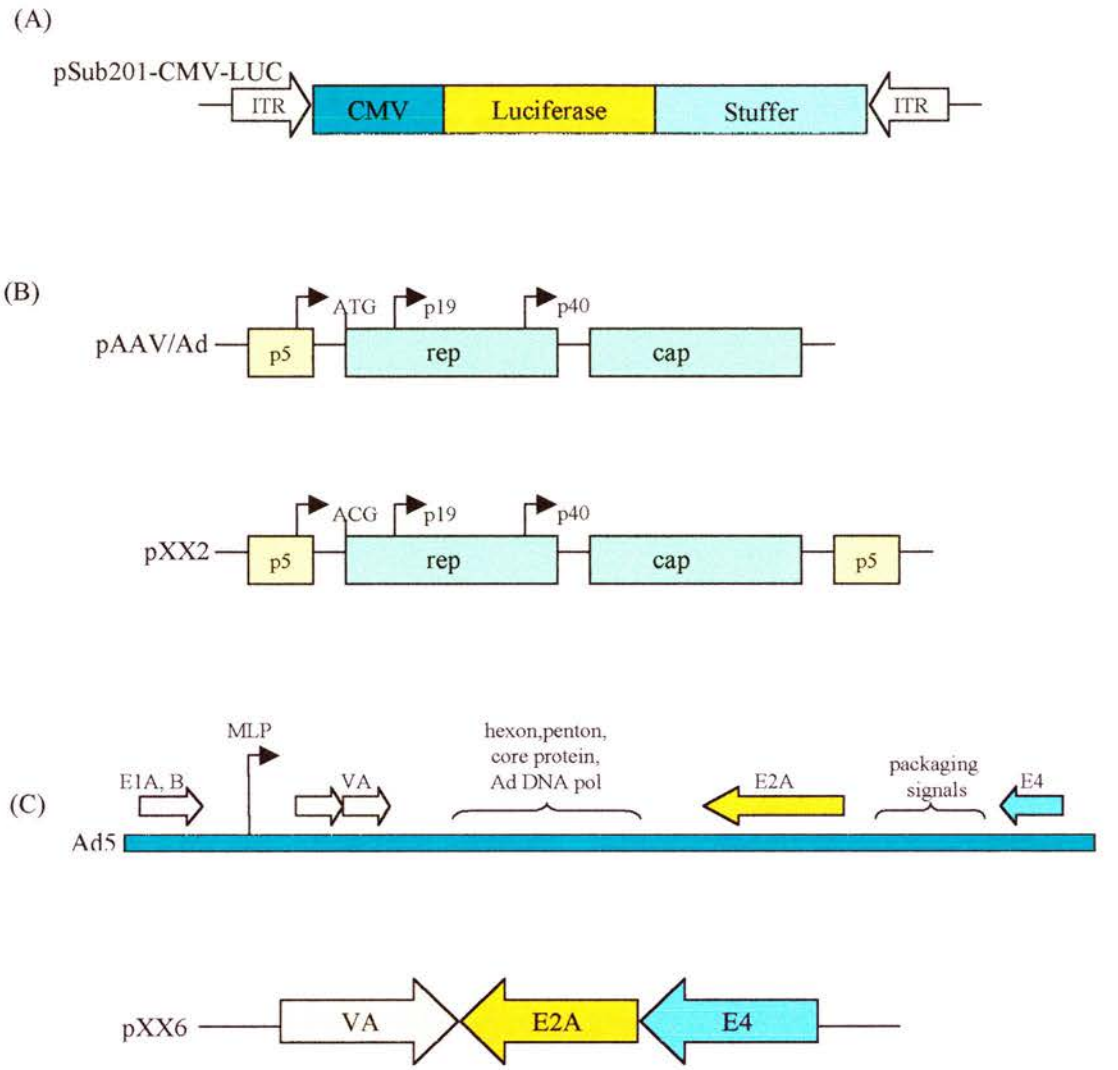

Figure 3.2: Plasmids used for rAAV optimisation experiments. (A) AAV vector pSub201-CMV -LUC contains the CMV promoter driving the luciferase reporter gene and stuffer DNA flanked by the AAV ITRs. This superfluous DNA is neccesary to ensure fragment is similar in length to the AAV genome (4.5 kb) for successful packaging. (B) AAV helper plasmid pXX2 differs from pAAV/Ad in that it has a mutation in the start codon of *rep78/68* gene which reduces Rep78/68 synthesis. It also has an additional p5 promoter downstream of *cap* gene. (C) Ad helper plasmid compared to Ad DNA sequences shows that only the sequences VA, E2A and E4 essential for supplying AAV helper function are retained. E1A and E1B are also essential but are complemented when plasmid transfected into 293 cells. Figures (B) and (C) are adapted from Xiao *et al.* (1998).

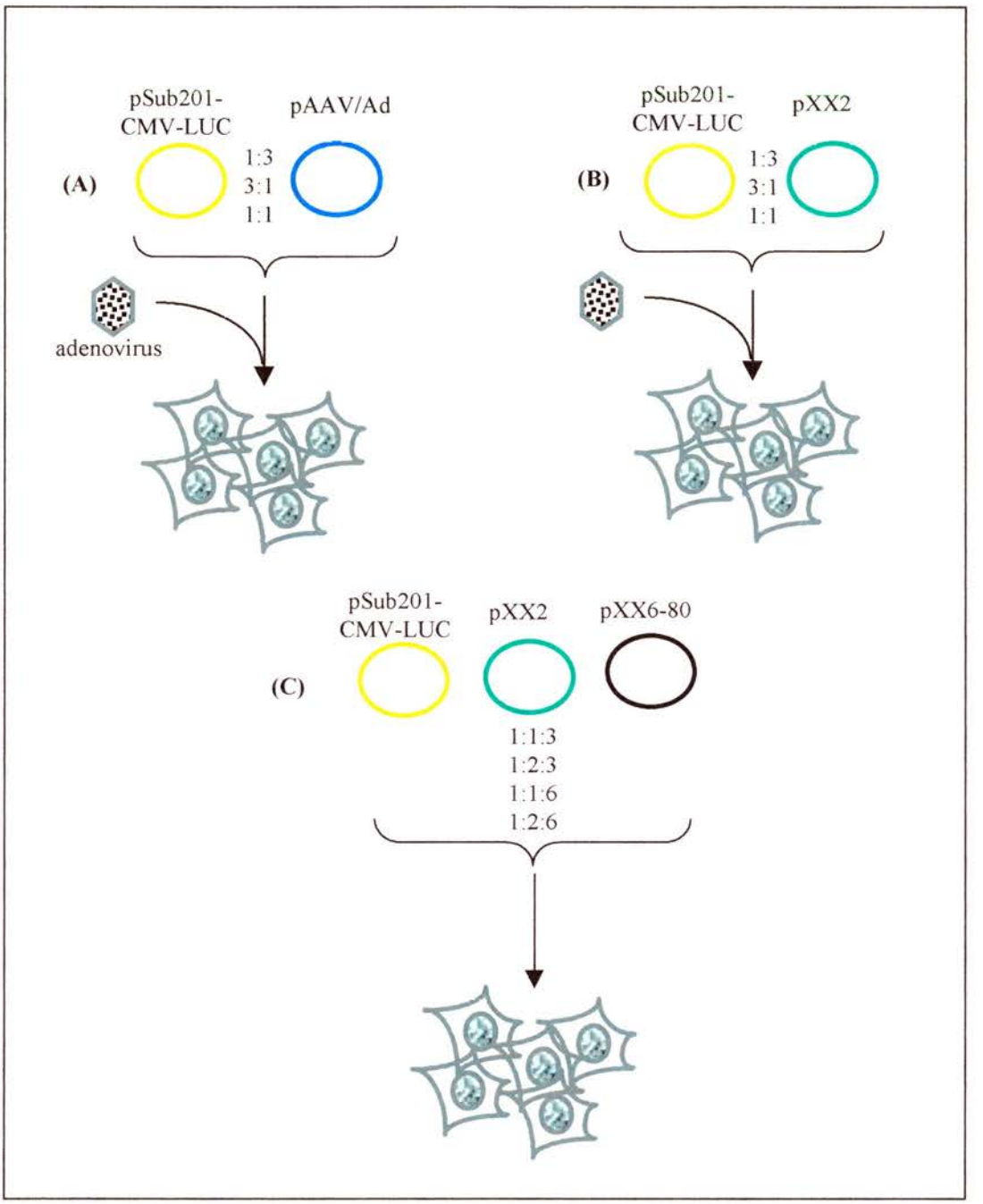

Figure 3.3: Experiments performed for the optimisation of rAAV production. All cells were transfected with pSub201-CMV-LUC, in addition to (A) pAAV/Ad followed by infection with adenovirus, (B) pXX2 followed by infection with adenovirus and (C) pXX2 together with pXX6-80. Different ratios of pSub201-CMV-LUC to AAV helper plasmids were also examined.

Helper	Ratio of pSub-CMV-LUC: AAV helper	Titre (replicative particles/ml)
pAAV/Ad	1:3	$1.5x10^6$
	3:1	$4x10^6$
	1:1	$2x10^6$
pXX2	1:3	$6x10^6$
	3:1	$1x10^7$
	1:1	$1x10^7$

Table 3.4: Titres of rAAV-CMV-LUC generated when AAV helper plasmids pAAV/Ad or pXX2 are used in conjunction with adenovirus. Different ratios of pSub-CMV-LUC: AAV helper plasmid was also compared.

Ratio pSub.CMV-LUC:pXX2:pXX6-80	Titre (replicative particles/ml)
1:1:3	$7x10^6$
1:2:3	$7x10^7$
1:1:6	$5x10^7$
1:2:6	$2x10^7$

Table 3.5: Titres of rAAV-CMV-LUC generated when the three plasmid system (Xiao *et al.* ,1998) is used. Different ratios of pSub201-CMV-LUC: pXX2:pXX6-80 were also compared.

3.2.3 Summary of optimisation experiments

Initially in the laboratory, rAAV stocks were generated using the original protocol described by Samulski *et al.* (1989) (section 1.4.7). rAAV is now generated by transfecting 293 cells with plasmids pSub201, pXX2 and pXX6-80 at a ratio of 1:2:3. Transfection is achieved using Effectene transfection reagent at a DNA:Effectene ratio of 1:25. Cells are harvested at 72 hours post-transfection and rAAV stocks titred (figure 3.4). Although it later became apparent that it may be necessary to further purify this lysate further (chapter 4), it was believed at the time this work was performed that this crude cell lysate should be suitable for experiments involving infection of cultured DRG neurons. This method of rAAV production avoids contamination of stocks by adenovirus and adenovirus cytotoxic proteins. This is an important consideration, as it is known that high amounts of contaminating adenovirus are toxic to the cultured DRGs (Revah *et al.*, 1996). In addition, the presence of contaminating adenovirus may influence results when studying PPT-A promoter regulation (see chapter 4).

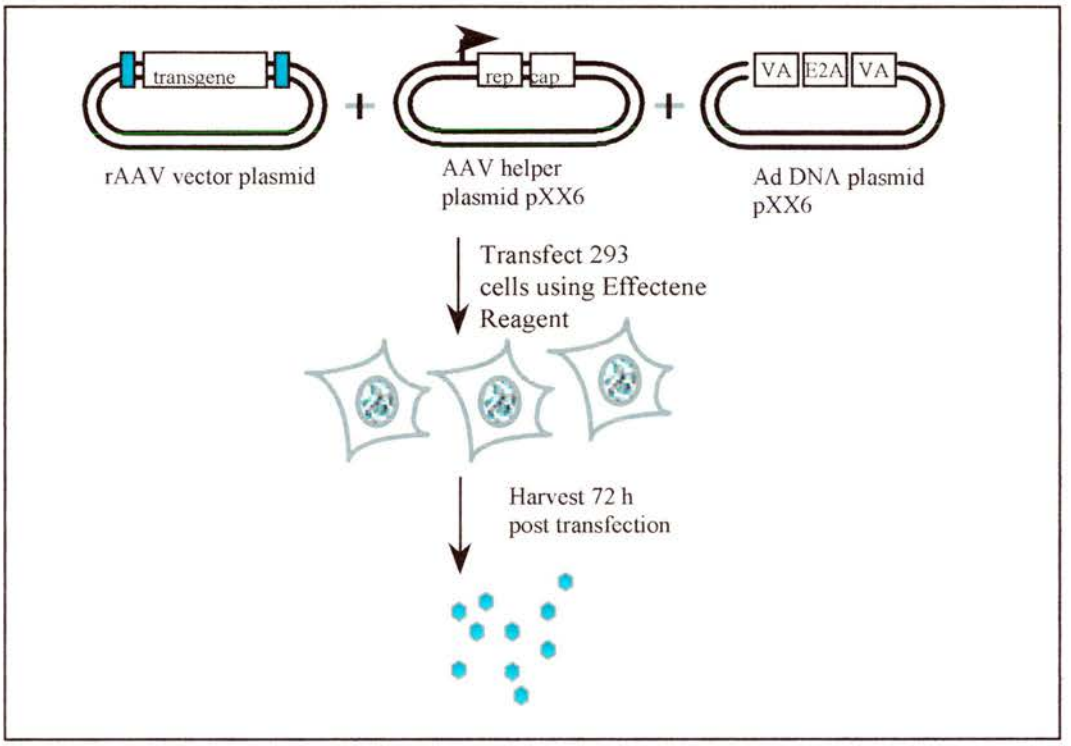

Figure 3.4: Optimised protocol for the production of rAAV vectors. 293 cells are transfected with AAV vector, AAV helper pXX2 and Ad plasmid pXX6 at a ratio of 1:2:3 μg DNA respectively. Cells are transfected using Effectene transfection reagent and harvested 72 h post-transfection.

3.3 Generation of DRG neuronal cultures

Cultures of DRG neurons were obtained from both neonate (section 2.2.7.2) and adult rats (section 2.2.7.1) for use in this study. DRG were removed from all spinal levels of the animals. To achieve cultures that were enriched for neuronal cell populations, adult neuronal preparations had an additional pre-plating step. The dissociated DRG were plated onto 0.5 mg/ml poly-D-ornithine coated plates overnight to allow the non-neuronal cells to adhere tightly while the neurons only loosely adhered. The neurons were washed off and then plated onto polyornithine and laminin coated coverslips (figure 3.5). Only preparations from adult DRG were treated in this way as adult rats appear to be surrounded by excessive connective tissue, which is difficult to remove and therefore increases the likelihood of contaminating the cell culture with epithelial and other nonneuronal cells. If necessary, cultures were treated with arabinose cytosine to limit the growth of nonneuronal cells. Arabinose cytosine disrupts DNA synthesis and therefore dividing cells are specifically killed. The significant growth of nonneuronal cells is particularly a problem for rAAV transduction experiments as cells must be maintained in culture for 7 days post-infection as this time scale is required for gene expression from AAV vectors (Afione *et al.*, 1999; personal observations).

Since this model of cultured DRG neurons was used in all subsequent studies, it was necessary to establish the cell types that are generated and survive in culture when DRG are processed in this way.

3.3.1 Neurofilament, IB4 and SP immunostaining of adult rat DRG cultures

As described in section 1.3, sensory neurons can be divided into three broad subgroups that are associated with specific neuronal functions and are responsive to distinct growth factors. It was therefore important to determine if each of the populations were present in these DRG cultures. Briefly, the large cell diameter (> 30 μm) population make up 30% of DRG neurons and can be identified using antibodies to neurofilament. The other two groups, the peptidergic and non-peptidergic populations have small cell diameters (< 30 μm) and make up 40 and 30% of DRG neurons respectively. The peptide containing group can be identified by using antibodies against the neuropeptide substance P whereas the non-peptidergic group possess cell surface glycoconjugates which can be identified by binding isolectin IB4 (Lewin, 1992). It is important to note that these percentages represent the normal state of DRG cells and under certain circumstances, such as inflammation and nerve damage the expression of these markers may change (Hokfelt *et al.*, 1994).

Neurofilament (NF), substance P (SP) and IB4 immunostaining was analysed individually in cultured DRG neurons (section 2.2.8). In control experiments the primary antibody was omitted. Figure 3.6 shows that the NF marker was associated with the perikarya, dendrites, and axons of neuronal populations that are large in cell body diameter. IB4 immunostaining was mainly observed in the cell bodies of neurons that are small diameter (figure 3.7) and antibodies against SP (figure 3.8) showed staining in the cell bodies of small diameter neurons, together with a number of cells that may be medium sized diameter.

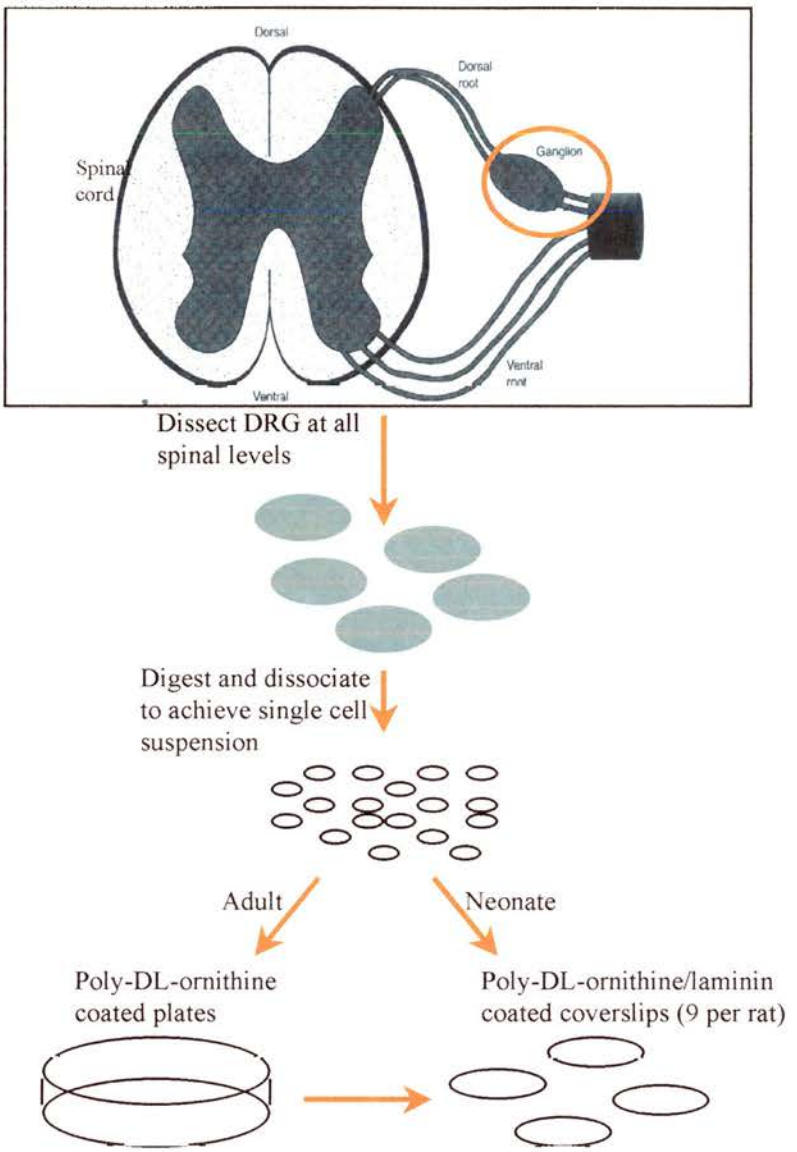

Figure 3.5: Generation of cultured DRG neurons. Dorsal root ganglia (DRG) are removed from all levels of the spinal cord and treated with collagenase to digest. Ganglia are mechanically dissociated to achieve single-cells and subsequently plated on coated coverslips. Neurons from adult rats undergo a pre-plating step to eliminate nonneuronal cells.

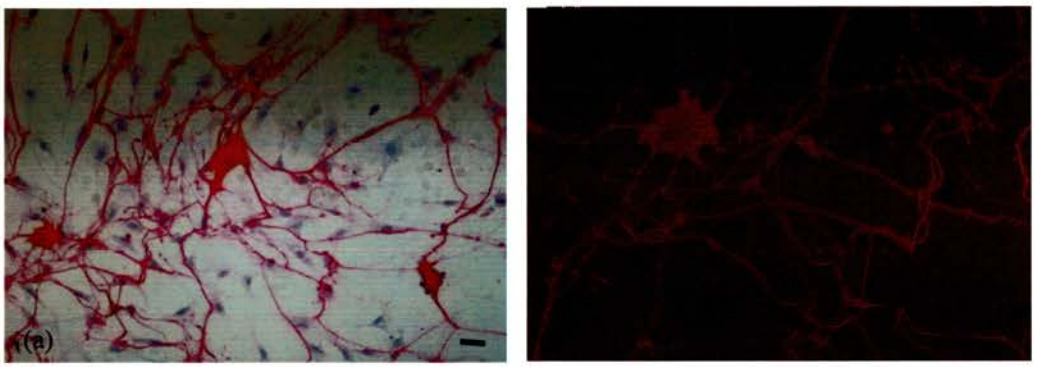

Figure 3.6: Neurofilament immunostaining of adult DRG cultures. Cells stained for NF are pink and (a) and (b) show both large and medium sized neurons are positive for NF.
Scale = (a) 50 μm and (b) 25 μm.

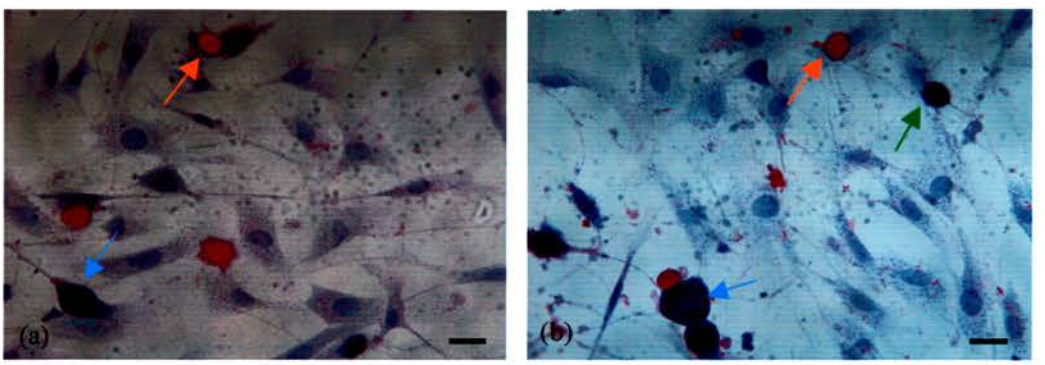

Figure 3.7: IB4 immunostaining of adult DRG cultures. A population of small cell neurons were positive for IB4 (red arrows) and are shown in both (a) and (b). Blue arrows highlight the large neurons which have not been IB4 labelled and the green arrow in (b) shows that some small neurons were not IB4 positive. Cells counterstained with haematoxylin are blue in colour.
Scale = 25 μm.

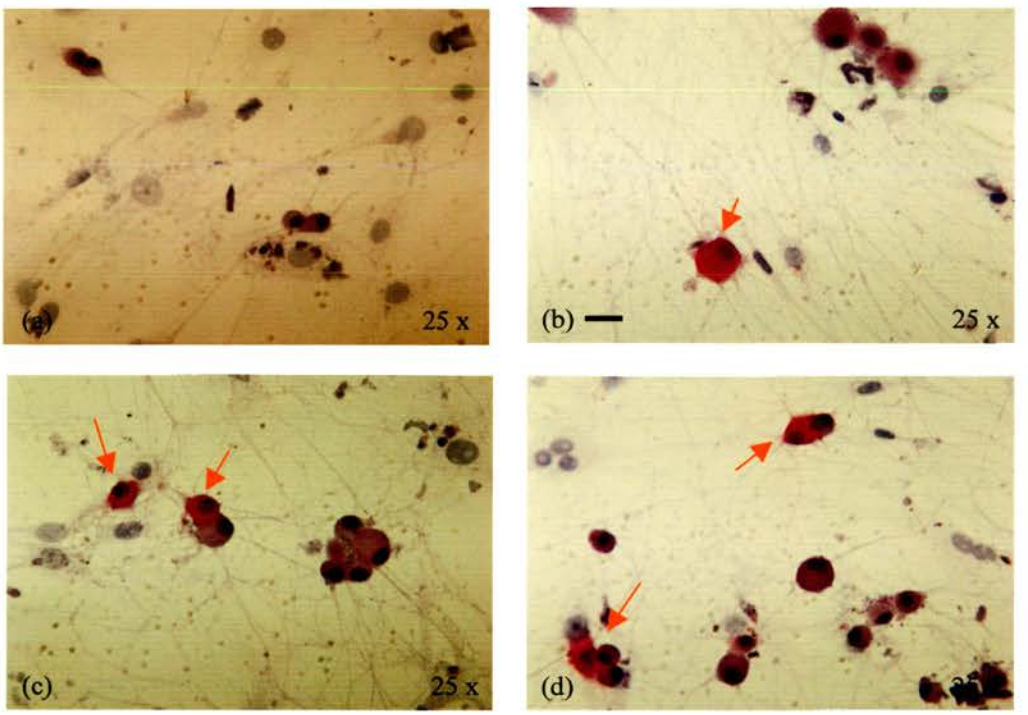

Figure 3.8: Adult DRG cultures immunostained for SP. Cells expressing SP are highlighted with the red arrows as expression is quite low and it difficult to distinguish SP positive cells from counterstained cells. Staining was mainly associated with small diameter cells (c) and (d) although staining was also observed in some medium neurons (b). For control experiments, SP antibody was omitted (a). Scale = 25 μm.

3.3.2 Discussion

These experiments show that the DRG cultures generated for these studies are highly heterogenous and immunostaining for SP, NF and IB-4 has identified different populations of DRG. This expression pattern is consistent with the consensus that the DRG is composed of three broad populations of neurons (Lawson, 1992) and these cultures contain members of each of the neurochemically defined populations of DRG cells. This is therefore a suitable model for investigating the transduction of sensory neurons by AAV. Furthermore, these experiments provide the basis for determining which neuronal populations support expression of the PPT-A promoter and will allow subsequent promoter regulation studies. Since it is known that these different populations are responsive to different stimuli, the promoter activity could be examined in individual cell populations in response to a variety of conditions and growth factors. Recently the technique of biolistic firing (also called particle-mediated transfection) into organotypic cultures has been employed for investigating PPT-A promoter regulation in cortical brain slices. This technique enabled plasmid constructs, containing the PPT-A promoter driving the GFP reporter gene, to be introduced into neurons and confocal microscopy was used to quantify single cell immunofluoresence (Walker *et al.*, 2000). This method of particle-mediated transfection can therefore enable analysis of neuronal gene promoters within single neurons in real time. Similar analysis using the AAV model for investigating PPT-A promoter regulation could provide invaluable information about expression of the PPT-A gene in DRG neurons.

3.4 Infection of DRG neurons with rAAV

It has been previously established that AAV can transduce cultured DRG neurons. DRG cultures were infected with rAAV vectors that contained the CMV or PPT promoter driving the luciferase reporter gene. Cultures were subsequently harvested and assayed for luciferase activity (Harrison *et al.*, 1999). However, this method does not allow the transduction efficiency to be established or to determine what cell populations are transduced in DRG cultures.

To address these queries, rAAV particles that contain the CMV or PPT (spanning base pairs –865+92) promoter driving the GFP reporter gene were used to infect the cultured DRGs. These preparations termed AAV-CMV-GFP and AAV-PPT-GFP were used to investigate which cell types support PPT promoter activity and therefore express GFP. Neurons were identified by their characteristic morphology and nonneuronal cells appeared to be either broad flattened or small spindel shaped cells. DRG cultures were initially infected with $2.5x10^6$ particles of AAV-CMV-GFP and AAV-PPT-GFP (section 2.2.10.1). Approximately 7 days post-infection, GFP expression was observed. The number of cells expressing GFP and the intensity was greater in those cultures transduced by AAV-CMV-GFP compared to those infected with AAV-PPT-GFP. Figure 3.9 shows GFP expression 7 days post-infection of DRGs with AAV-CMV-GFP, however the fluorescent microscope and photography equipment available was not sensitive enough to capture the images of those cells expressing PPT-GFP therefore it was decided to use antibodies to detect GFP.

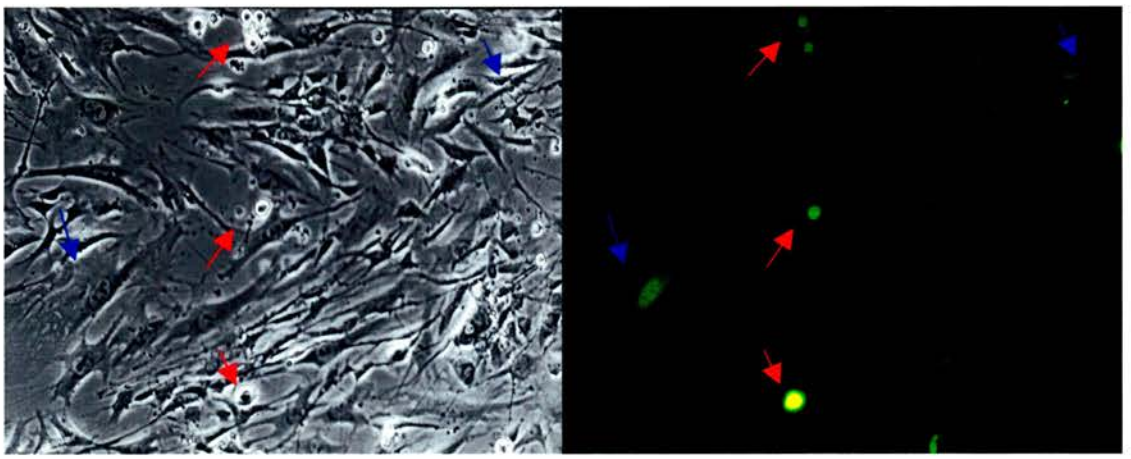

Figure 3.9: GFP expression in cultured adult DRG neurons. Cells were infected with rAAV-CMV-GFP and 7 days post infection GFP expression was observed. Both neuronal and nonneuronal cells were GFP positive. Neurons are identified by their characteristic morphology (red arrows) and nonneuronal cells are highlighted by the blue arrows.

3.4.1 Dose dependent transfer of GFP gene expression

Cultures of adult DRGs containing both neuronal and nonneuronal cells were infected with increasing concentrations of AAV-CMV-GFP and AAV-PPT-GFP. The Gene Therapy Services unit at Genethon generated the rAAV preparations for these studies (section 2.2.9.7). This service produced extremely pure, high titre rAAV stocks that could not be feasibly generated in this laboratory and therefore allowed high doses of rAAV to be used for infection. In addition, these Genethon samples could be used as standard for comparison with our own rAAV preparation.

Approximately 500-1000 adult DRG neurons were grown on 9 mm coverslips (section 2.2.7.2) and 0, 4.5 x 10^5, 1.8 x 10^6, 4.5 x 10^6 and 9 x 10^6 infectious particles of AAV-CMV-GFP were used for infection of these cultures (section 2.2.10.1). Maximum GFP expression was observed 7 days after infection. Cells were fixed and subject to GFP immunostaining (section 2.2.10.4). In all infections both neurons and nonneuronal cells expressed GFP and the efficiency of gene transfer to neurons was related to the concentration of virus (figure 3.10). Cells infected with 4.5 x 10^5 particles showed very low levels of expression (figure 3.10 B). The cell numbers expressing GFP was also quite low in those infected with 1.8 x 10^6 particles (figure 3.10 C and D). When 4.5 x 10^6 particles were used there is a marked increase in the number of positive cells and almost all neurons and nonneuronal cells appear to be stained for GFP (figure 3.10 E, F and G). In these circumstances the transduction efficiency of cell types in the cultures cannot be specifically defined, as it is highly likely that the number of nonneuronal cells increased with cell growth throughout the time course of the experiment. However, it is clear that transduction efficiency of neurons is almost 100% when 4.5 x 10^6

particles were used for infection. In addition, GFP expression and the intensity of staining is greater in neurons than in the noneuronal cells. When 9 x 10^6 particles are used (figure 3.9 H, I and J) there is not a marked difference compared to those cultures infected with 4.5 x 10^6 particles. This suggests that future experiments should employ at least 4.5 x 10^6 particles to achieve infection of all neurons in a typical 9 mm coverslip culture (approximately 500-1000 neurons). Therefore, this can be calculated at $4.5x10^3$ rAAV infectious particles per neuron. Although this appears to be a very high number of virus particles per cell, it is important to remember that the method by which rAAV particles are usually titred (replication centre assay) does not always relate specifically to the number of transcriptionally active rAAV particles. Rather it describes the number of replication competent rAAV particles, which can often be 100 fold higher than the number of actual transducing rAAV particles (section 1.4.9).

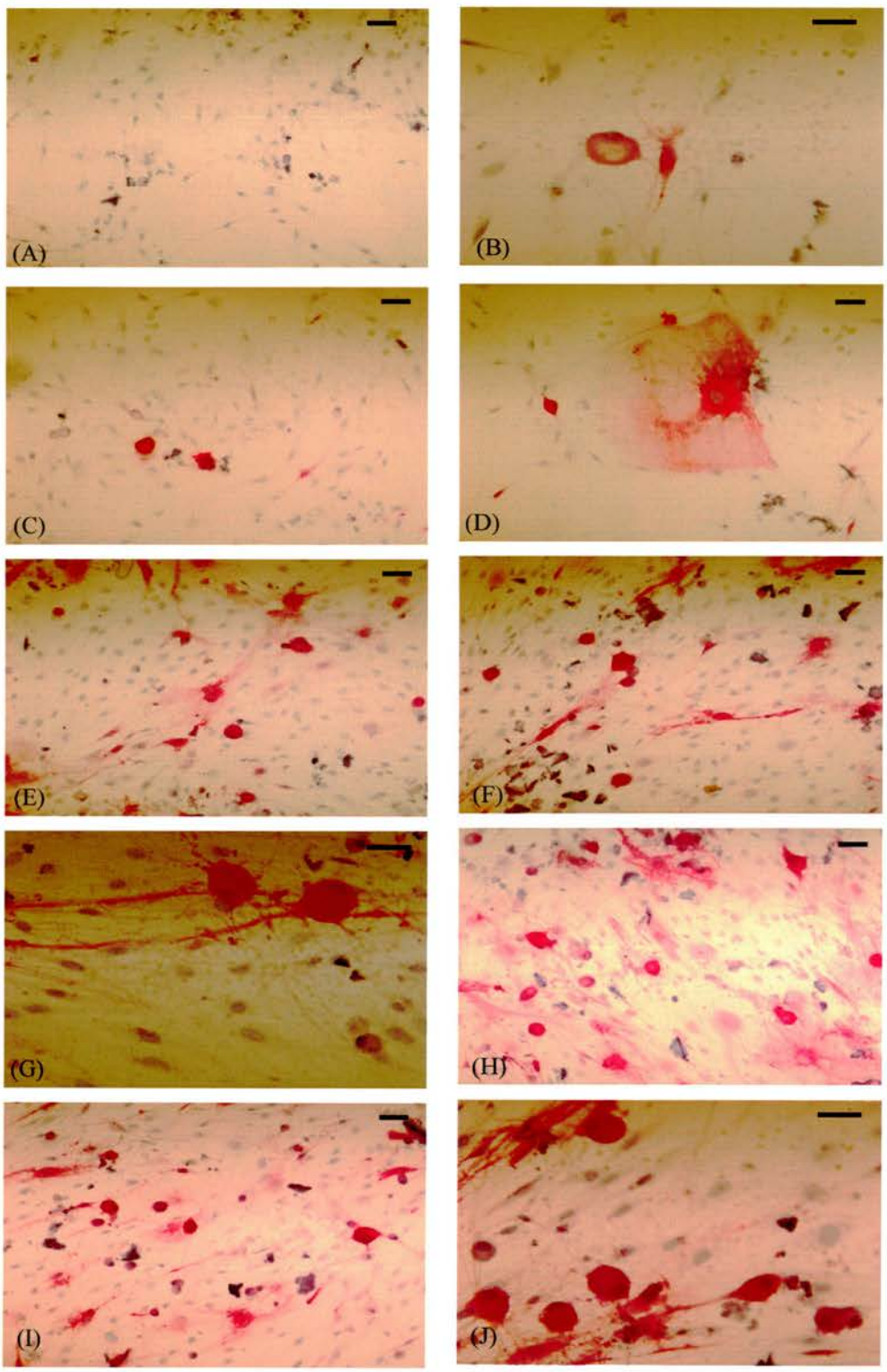

Figure 3.10: Adult DRG infected with increasing concentrations of AAV-CMV-GFP and immunostained for GFP 7 days post infection. Cells positive for GFP are pink in colour, and were infected with no virus (A), 4.5 x 10^5 particles (B), 1.8 x 10^6 particles, (C) (D), 4.5 x 10^6 particles (E) (F) (G) and 9 x 10^6 particles (H), (I) (J). Scale = 50 μm 10 x mag (A), (C), (D), (E), (F), (H) and (I); 25 μm 25 x mag (B), (G) and (J).

3.4.2 Population of neurons which support PPT promoter activity

Adult DRG cultures were infected with increasing concentrations of AAV-PPT-GFP (which were also generated by Genethon) as described above. Again 0, 4.5 x 10^5, 1.8 x 10^6, 4.5 x 10^6 and 9 x 10^6 infectious particles were used to infect approximately 500-1000 DRG neurons on 9 mm coverslips. GFP expression was observed 7 days post-infection and cultures were immunostained for GFP expression (section 2.2.10.4).

GFP expression driven by the PPT promoter was observed in neurons and nonneuronal cells (figure 3.11). When 4.5 x 10^5 infectious particles was used for infection, only a small number of cells were GFP positive (data not shown). When the amount of virus was increased to 1.8x10^6, an increase in GFP expression was observed, although expression from the AAV-PPT-GFP infected cells was low compared to those cultures infected with an equivalent number of AAV-CMV-GFP particles (figure 3.11 A, B). Infection with 4.5 x 10^6 and 9 x 10^6 infectious particles of AAV-PPT-GFP resulted in approximately 30 to 50 GFP positive cells per coverslip and those infected with 9 x 10^6 infectious particles showed more intense staining (figure 3.11 C-H). The proportion of GFP expressing cells is much less (approximately 10% expression) than those cultures infected with AAV-CMV-GFP (almost 100% expression; compare figures 3.10 and 3.11). Cells infected with 4.5 x 10^6 and 9 x 10^6 infectious particles of AAV-CMV-GFP reach a saturation point whereby all cells were expressing GFP. This suggests that all the neurons have been transduced by AAV-PPT-GFP but only a proportion of these cells supported GFP expression driven by the PPT promoter. Efficiency of gene transfer to DRG neurons is therefore related to the concentration of virus and is dependent on promoter types.

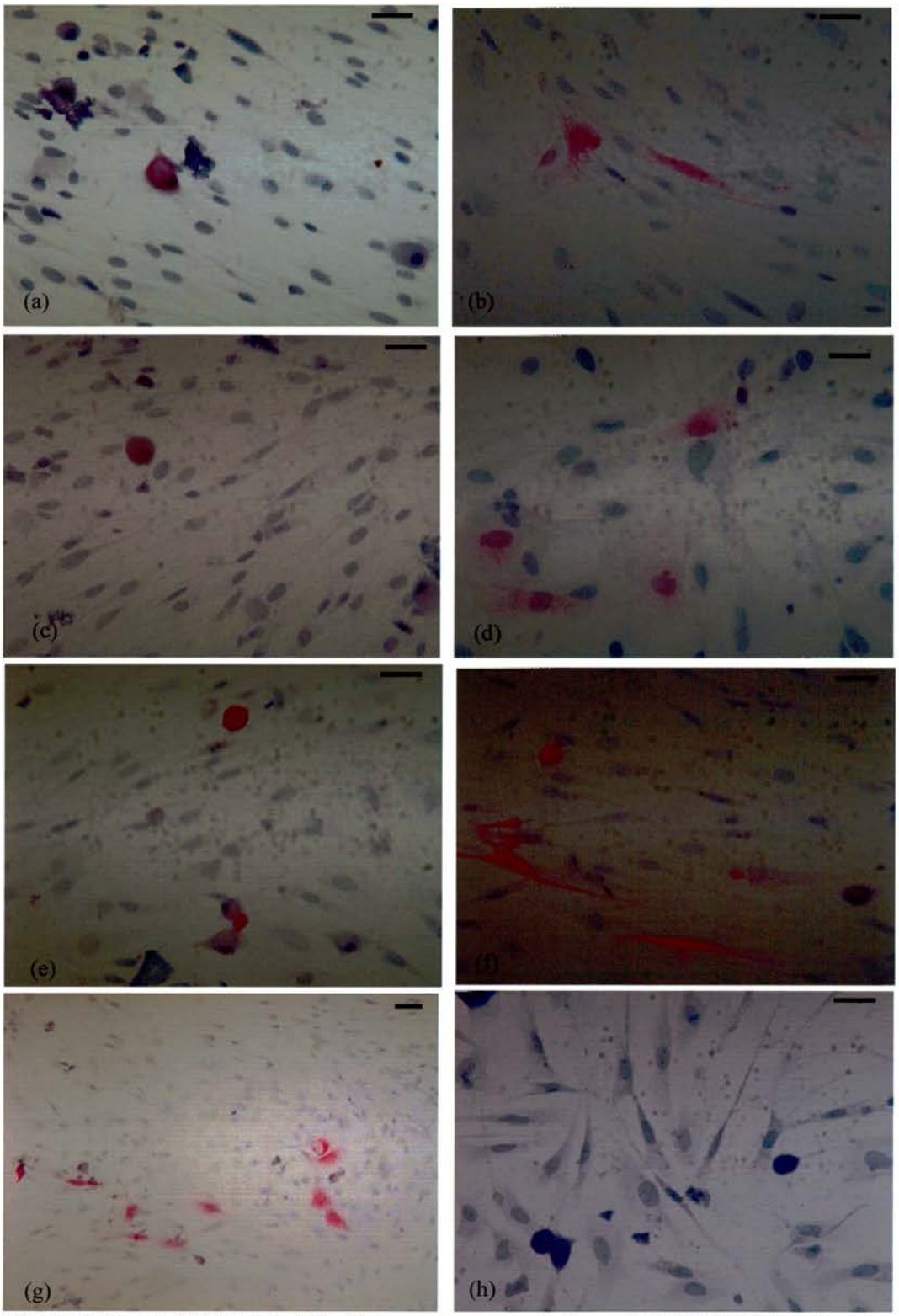

Figure 3.11: Adult DRG infected with increasing concentrations of AAV-PPT-GFP and immunostained for GFP 7 days post-infection. Cells positive for GFP are pink in colour and were infected with $1.8x10^6$ particles (a), (b), $4.5x10^6$ particles (c), (d), $9x10^6$ particles (e), (f), (g) and no virus (h). Scale = 25 μm 25 x mag (a)-(f) and (h); 50 μm 10 x mag (g).

3.4.3 Discussion

This study has demonstrated AAV-mediated gene transfer to cultured DRG neurons by both AAV-CMV-GFP and AAV-PPT-GFP vectors. It has defined several of the parameters that may be important for efficient transduction and the cell populations that are transduced by AAV. In addition, this study has implicated the cell types that might support reporter gene expression when the PPT promoter is utilised.

It was found that both AAV-CMV-GFP and AAV-PPT-GFP express GFP in both neurons and nonneuronal cell types. The number of GFP positive neurons increased as a greater number of AAV-CMV-GFP virus particles were added and when 4.5×10^6 infectious particles of AAV-CMV-GFP was used for infection, approximately all neurons were expressing GFP. This indicates a dose-dependent pattern of AAV mediated gene expression, with the possibility of reaching a maximum of 100% efficiency at high doses of AAV.

Cultures infected with AAV-PPT-GFP showed less expression both in the number and intensity of GFP positive cells. Since similar doses of AAV-CMV-GFP showed 100% transduction efficiency, it may be the case that all neurons were transduced by AAV-PPT-GFP but only a limited population of cells in culture support expression of the PPT promoter therefore PPT promoter expression is restricted compared to the CMV promoter. To determine if this may be the case, it would be necessary to confirm the presence of viral DNA in the cells.

The PPT promoter has been described as supporting neuron specific reporter gene expression when delivered as a rAAV vector to DRG cultures (Harrison *et al.*, 1999). In this study by Harrison *et al.*, expression of the luciferase reporter gene was

detected in DRG cultures but not in clonal cell lines however, this assay did not allow the PPT expression to be visualised directly. In another study, analysis of PPT promoter activity in DRG neurons was performed by microinjection of plasmid DNA (containing the PPT promoter driving the β-galactosidase reporter) directly into neurons (Mulderry *et al.*, 1993). This study did allow the DRG neurons expressing the PPT promoter to be identified but did not investigate PPT expression in cell populations other than the microinjected neurons. In agreement with the work presented here, Mulderry *et al.* did find that expression was restricted to only a small percentage of the neurons injected. In addition, Mulderry *et al.* revealed that expression of the PPT constructs was not selective for DRG neurons containing immunoreactive SP. This indicated that the promoter can support expression in neuronal cells that do not always express the endogenous PPT gene. To test cell type-specificity of expression of PPT constructs, Mulderry *et al.* also injected the PPT constructs into nonneuronal cells of DRG cultures and low levels of PPT promoter activity was observed.

This is the first study performed that examines PPT promoter activity directly in all cell populations of cultured DRG neurons. When DRG cultures were infected with AAV-PPT-GFP, the PPT promoter can support expression in some nonneuronal cells. Analysis of plasmids containing this promoter fragment driving the GFP reporter gene in cortical brain slices also observed GFP expression in nonneuronal cell populations (Walker *et al.*, 2000). PPT gene expression in the noneuronal population of cells is known to occur and astrocytes can express SP (Too *et al.*, 1994; Lin, 1995).

Therefore the work presented here suggested that the PPT promoter is active in nonneuronal cells and this has been previously observed (Mulderry *et al.*, 1993; Walker *et al.*, 2000). An extremely important consideration, which may account for these observations, is that the presence of such high copy numbers of AAV vectors or plasmid DNA containing the PPT promoter in nonneuronal cells might act to overcome repression of the PPT-A promoter. For example, plasmids containing the PPT promoter do not normally support reporter gene expression in PC12 cell lines (Mendelson *et al.*, 1995). However, when this reporter construct was cotransfected into PC12 cells together with a plasmid containing multiple copies of the postulated repressor binding site, expression of the PPT promoter was observed (Mendelson *et al.*, 1995). Therefore the presence of multiple copies of the repressor binding site acted to titrate the repressor from the reporter gene allowing the PPT promoter to support reporter gene expression.

Together this work provides extremely important information about the infection of DRG neurons with AAV. This is important if this virus vector is to be used in the study of investigating transcriptional regulation of the PPT promoter and other neuron-specific genes. Furthermore, these studies have formed the basis for future experiments to define specifically the cell populations that can support expression of the PPT-A promoter. This could be achieved by performing immunostaining of infected cells to identify the specific cell types that are expressing GFP and will ultimately lead to a greater understanding of the PPT-A gene regulation.

CHAPTER 4: REGULATION OF THE PPT-A PROMOTER IN CULTURED DRG NEURONS

4.1 Introduction

The aim of this study was to investigate the transcriptional regulation of the PPT-A promoter in cultured DRG neurons. DNA binding studies have allowed models of transcriptional regulation of the PPT-A promoter to be proposed (section 1.2.3) and these can be tested by the introduction of reporter gene constructs into DRG neurons in culture or clonal cells lines. However functional analysis of the PPT-A promoter has been greatly hindered due to the lack of clonal cells lines that express endogenous PPT-A or support reporter gene expression driven by the PPT-A promoter, together with the difficulties associated with transfection of DRG neurons. This study set out to overcome these problems associated with functional analysis of the PPT-A promoter in DRG neurons. This was achieved by using rAAV as a vector for transduction of DRG neurons. Regulation of the promoter was investigated in two ways. Firstly, expression of promoter constructs that contained varying lengths of the PPT-A promoter was analysed. It was hoped that these studies would define functional elements involved in PPT-A promoter activity in cultured sensory neurons. Secondly, the effect of growth factors on the PPT-A promoter was studied. Plasticity of PPT-A gene expression is an established occurrence and expression is induced or repressed by a variety of stimuli (Hokfelt *et al.*, 1994). It was therefore hoped that these studies would provide insight into PPT-A gene regulation and the signal transduction pathways involved in mediating these effects.

4.2 Regulation of PPT-A promoter activity: Deletion analysis

4.2.1 Background

As previously stated functional analysis of the PPT-A promoter has been greatly hindered. Initial functional studies employed the technique of microinjection of plasmid DNA into cultured DRG neurons. Only a small number of studies have been carried out in this way as this procedure is extremely time consuming and allows the analysis of only a small number of neurons (section 1.2.3). Studies have also focused on the role of individual promoter elements in cultured DRG neurons and HeLa and PC12 cell lines. These cell lines cannot support the expression of the entire PPT-A proximal promoter due the presence of a dominant repressor (section 1.2.3). Subsequently isolated promoter elements were linked to heterologous promoters to drive reporter gene expression (section 1.2.3). These studies have determined whether specific motifs have the potential to be activator or repressor elements, allowing models of promoter regulation to be proposed. However it is not clear if these sites have the same function in isolation as they do when in context of the entire promoter. It is likely that transcription factor binding sites may be differentially regulated in cultured DRG neurons compared to cell lines such as HeLa and PC12 that do not express endogenous PPT-A. More recently, two cell lines expressing endogenous PPT-A have been used for functional analysis of PPT-A promoter and have established transcription factor binding sites that are involved in transcription of the proximal promoter (Fiskerstand *et al.*, 1999). The use of these cell lines allow potential transcriptional mechanisms to be deciphered before performing analysis in DRG neurons, in which only a small number of experiments are feasible. However it is important to appreciate that the plasticity of the PPT-A

expression in DRG neurons means that regulation of the PPT-A gene could differ between cell lines that express endogenous PPT and the DRG neurons. PPT-A expression is regulated differently between different neuronal types, for example sensory and sympathetic neurons (Rao *et al.*, 1993), and even within populations of sensory neurons, PPT-A expression is differentially regulated (Verge *et al.*, 1995). Consequently, the factors involved in modulating expression of the PPT-A gene are extremely complex. This study set out to investigate the regulation of the PPT-A gene expression in cultured adult and neonate rat DRG neurons. Neurons were transduced by infection with AAV recombinant virus particles containing 5'and 3' flanking DNA sequences linked to the luciferase reporter gene to study promoter expression. In addition, the role of a specific 'E box motif' in PPT gene expression was also investigated in adult DRG neurons. Previous functional studies in PC12 and HeLa cells have implicated this element as an important regulator of PPT-A promoter activity.

4.2.2 Results

Previous work has shown that high levels of reporter gene activity is directed by promoter fragment -865 to +92 (relative to the transcriptional start site) and the majority of putative response elements of interest are contained in this proximal promoter fragment (section 1.2.3). To identify regulatory regions within this span of the PPT-A promoter, pSub201-derived constructs were generated that contained different lengths of the 5'-end of the PPT promoter driving luciferase as a reporter gene (section 2.2.3.1). In addition a construct containing the promoter fragment spanning base pairs -865 to +447 was generated, as the region spanning +92 to +447

has been shown during the course of this thesis to contain important transcription factor binding sites (Fiskerstrand *et al.*, 2000). These constructs were then used for the generation of recombinant AAV vectors (section 2.2.9.4) and the resulting vectors were termed AAV-865+447-LUC, AAV-865+92-LUC, AAV-671+92-LUC, AAV-484+92-LUC and AAV-47+92-LUC (figure 4.1). Unless otherwise stated, all rAAV preparations were generated using the three-plasmid optimised protocol described in section 3.2. Cultured DRG neurons from both adult and neonate rats were infected with these AAV vectors (section 2.2.10.1). The infected cells were maintained in culture for 7 days and then harvested by lysis. The cell extracts were then assayed for luciferase activity (section 2.2.10.3). All experiments were performed in the presence of NGF to allow equivalent comparison of promoter activity in DRGs from adult and neonate rats. Although cultured adult DRGs can be maintained in the absence of growth factors, neonate DRG cultures require NGF for survival (Lindsay, 1988).

4.2.2.1 Activity of PPT-A promoter constructs in adult DRG cultures

Due to the limited cell numbers obtained in generating adult DRG cultures, only a maximum of three promoter fragments could be compared in any one experiment. Therefore numerous experiments were carried out to compare the activities of different promoter fragments in each adult DRG experiment (table 4.1). To get a broad understanding of PPT-A promoter expression, the activities of two promoter fragments were compared. In particular, each promoter fragment activity was related to that of the minimum promoter fragment -47+92 (table 4.2 and figure 4.2).

AAV-865+447-LUC showed the highest expression levels (100%) (table 4.1) and promoter activity was induced an average of 60 fold compared to the minimum promoter fragment AAV-47+92-LUC (table 4.2). Expression was reduced 2 fold (50%) when the PPT-A promoter sequence was deleted to create fragment -865 to +92 compared to -865+447. Levels of expression from the -865+92 construct were approximately 30 fold greater than the minimum promoter fragment therefore showed 50% more activity than –47+92 (table 4.2). Further 5' deletions beyond –865+92 to leave fragments -671+92 or -484+92 caused a further reduction in expression (table 4.1). Promoter fragment -671+92 and -484+92 directed equivalent levels of expression (approximately 10%) (table 4.1), showing an average 6 fold increase in promoter activity compared to the minimum promoter fragment (table 4.2). These results are summarised in figure 4.2.

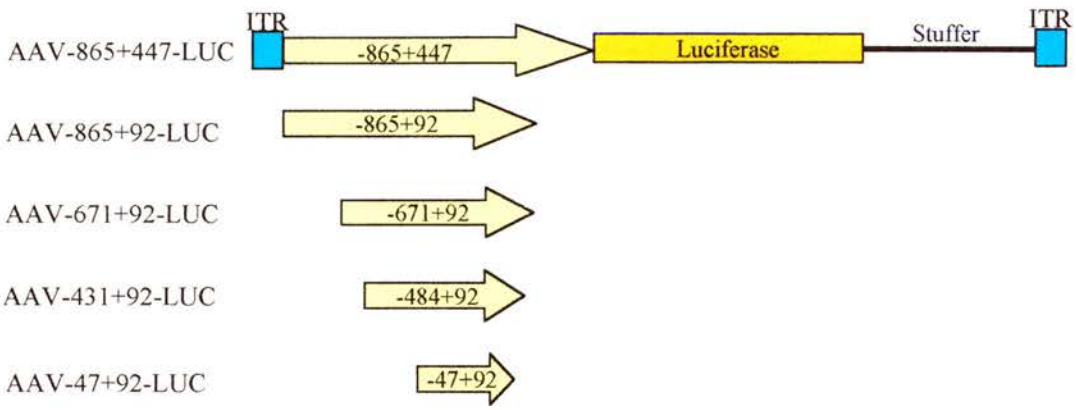

Figure 4.1: AAV recombinant virus generated for PPT-A promoter analysis. Figure shows AAV vectors containing the different promoter fragments driving the luciferase reporter gene. Important features of these AAV vectors are also shown including the ITRs and the stuffer fragment necessary to ensure transgene size is equal to that of AAV genome. These AAV vectors were used for infection of cultured DRGs from both adult and neonate rats.

	Expt	Promoter				
		-865+447	**-865+92**	**-671+92**	**-484+92**	**-47+92**
Luciferase values	1	-	2.387±0.220	0.493±0.144	0.401±0.062	-
	2	-	-	-	0.048±	0.013±
	3	1.069±0.482	0.532±0.298	0.022±	-	-
	4	-	41.68±5.04	-	12.56±1.298	1.59±0.219
	5	-	18.66±3.88	-	4.818±0.855	0.411±0.026
	6	32.46±18.92	3.807±0.342	-	-	0.28±0.047
	7	30.15±19.67	19.34±1.408	-	-	0.9±0.086
	8	26.02±5.702	10.64±1.405	-	-	0.84±0.298
	9	-	3.025±1.162	0.427±0.120	-	0.361±0.126
	10	58.83±6.675	35.83±3.567	-	-	1.384±0.011
	11	142.6±31.68	83.2±9.175	-	-	1.36±0.195
	12	-	0.180±0.058	0.041±0.012	-	-
	13	-	-	0.859±0.403	0.575±0.125	0.276±0.104
	14	-	-	1.188±0.618	1.756±0.573	0.132±0.04
	15	-	-	0.327±0.003	0.585±0.016	0.065±0.018
	16	0.852± 0.196	-	0.004±0.002	-	-
	17	0.276±0.078	-	-	0.004±0.001	-
	18	0.061±0.019	-	-	0.007±0.001	-

Table 4.1: Expression of PPT-A promoter vectors in adult DRG neurons. DRG cultures from adult rats were infected with AAV vectors containing the PPT-A promoter fragments -865+447, -865+92, -671+92, -484+92 and -47+92. Cells were maintained in medium + 100 ng/ml NGF for 7 days and then harvested and assayed for luciferase activity. The luciferase values shown are represented as mean and std. error of triplicate wells for each sample.

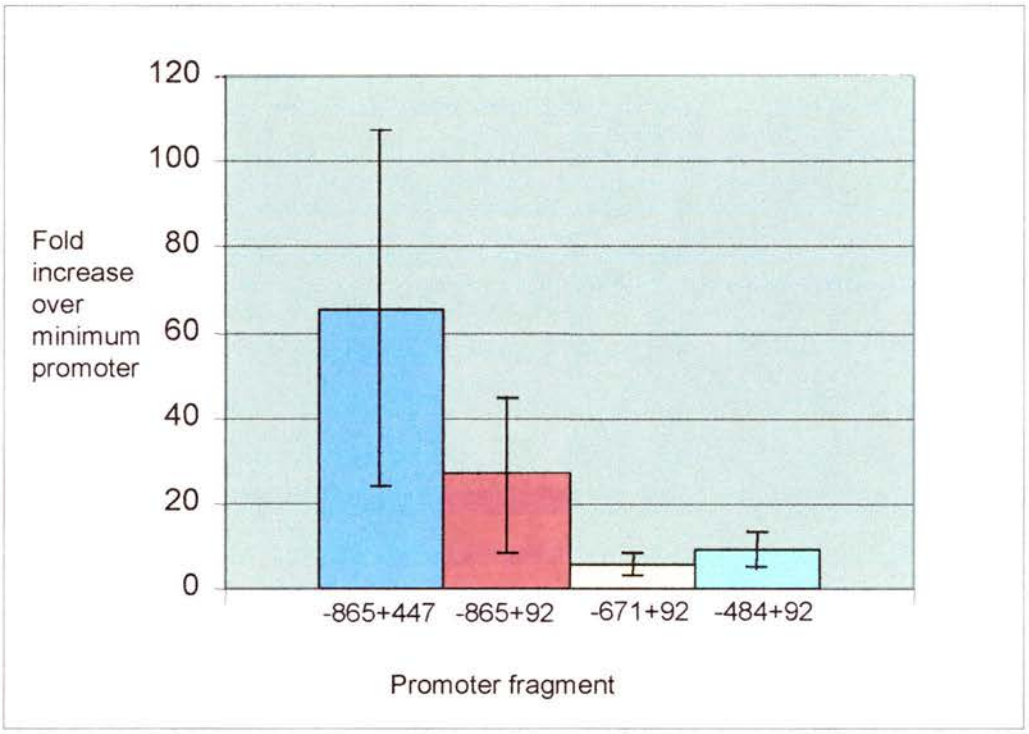

Figure 4.2: Expression of PPT-A promoter vectors in adult DRG cultures. DRG cultures were infected with AAV vectors containing the PPT-A promoter fragments -865+447, -865+92, -671+92, -484+92 and -47+92. Cells were maintained in medium + 100 ng/ml NGF for 7 days and then harvested and assayed for luciferase activity. Results are represented as fold increase induced by promoter fragments compared to expression from the minimum promoter -47+92. Data is expressed as mean ± SEM from three to eight independent experiments, whereby each experiment incorporated triplicate wells.

Expt	Luciferase values		Fold increase over minimum
	Promoter		
	-865+447	-47+92	
1	32.46 ± 18.92	0.28 ± 0.047	116
2	30.15 ± 19.67	0.9 ± 0.086	33.3
3	26.02 ± 5.702	0.84 ± 0.297	31.0
4	58.83 ± 6.675	1.384 ± 0.113	42.5
5	142.6 ± 31.68	1.36 ± 0.196	105
	-865+92	-47+92	
1	41.48 ± 5.04	1.59 ± 0.068	26.1
2	18.66 ± 3.88	0.411 ± 0.025	45.4
3	3.807 ± 0.341	0.28 ± 0.049	13.6
4	19.34 ± 12.97	0.9 ± 0.092	21.5
5	10.64 ± 1.406	0.84 ± 0.235	12.7
6	3.025 ± 1.162	0.361 ± 0.126	8.4
7	35.83 ± 6.214	1.384 ± 0.0114	25.9
8	83.2 ± 9.18	1.36 ± 0.195	61.2
	-671+92	-47+92	
1	0.859 ± 0.403	0.276 ± 0.104	3.1
2	1.188 ± 0.618	0.132 ± 0.094	9.0
3	0.327 ± 0.003	0.065 ± 0.018	4.8
	-484+92	-47+92	
1	12.56 ± 1.490	1.590 ± 0.219	7.9
2	4.818 ± 0.977	0.411 ± 0.026	13.4
3	0.575 ± 0.150	0.276 ± 0.103	2.1
4	1.756 ± 0.573	0.132 ± 0.040	13.3
5	0.585 ± 0.016	0.065 ± 0.018	9.0

Table 4.2: Comparison of promoter fragment activity to minimum promoter -47+92 in adult DRG cultures. Results from table 4.1 are represented as fold increase over the minimum promoter. That is the increase in expression induced by promoter fragments -865+447, -865+92, -671+92 and -484+92 compared with expression from promoter -47+92.

4.2.2.2 Activity of PPT-A promoter constructs in neonate DRG cultures

When the rAAV promoter vectors were analysed in neonate DRG cultures it was possible to compare a greater number of promoter fragments in one experiment. A greater number of neurons can be generated from neonate DRGs, which survive in culture. In addition, several animals can be used to produce one set of cultures for each experiment. Six separate experiments were performed in which expression from vectors AAV-865+92-LUC, AAV-484+92-LUC and AAV-47+92-LUC, were compared (table 4.3). In addition, four experiments included AAV-865+447-LUC and two experiments included AAV-671+92-LUC for comparison (table 4.3).

When the activity of promoter fragment -865+447 was examined, it was found to be approximately equal to that of -865+92. Both fragments showed the greatest promoter acvtivity being approximately 15 fold greater than the minimum promoter activity. Fragments –865+447 and –865+92 can therefore be described as possessing 100% promoter activity (table 4.4; figure 4.3). When sequences were removed to leave fragments -671+92 and -484+92, a large reduction in expression was observed. Promoter fragments -671+92 and -484+92 directed equivalent levels of reporter gene expression (table 4.3) and the activity of fragment -484+92 was approximately 50% (2 fold) less than that of the minimum promoter fragment. The increase and decrease in promoter activities over the minimum promoter are summarised in figure 4.3.

	Expt	Promoter				
		-865+447	**-865+92**	**-671+92**	**-484+92**	**-47+92**
Luciferase values	1	365±19.16	276±48.24	-	9.1±0.933	16.16±0.382
	2	213.5±13.13	109.9±14.68	-	5.763±0.049	11.57±0.174
	3	59.09±11.92	63.9±3.286	5.007±1.751	4.605±1.826	-
	4	162.8±47.04	150.2±4.017	9.175±0.787	7.244±4.849	27.85±11.20
	5	7666±1323	22.28±2.415	-	0.513±0.041	0.819±0.042
	6	999±70.65	14.65±3.805	-	0.551±0.076	1.481±0.110

Table 4.3: Expression of PPT-A promoter vectors in neonate DRG neurons. DRG cultures from neonate rats were infected with AAV vectors containing the PPT-A promoter fragments -865+447, -865+92, -671+92, -484+92 and -47+92. Cells were maintained in medium + NGF for 7 days and then harvested and assayed for luciferase activity. The luciferase values shown are represented as mean and std. error of triplicate wells for each sample.

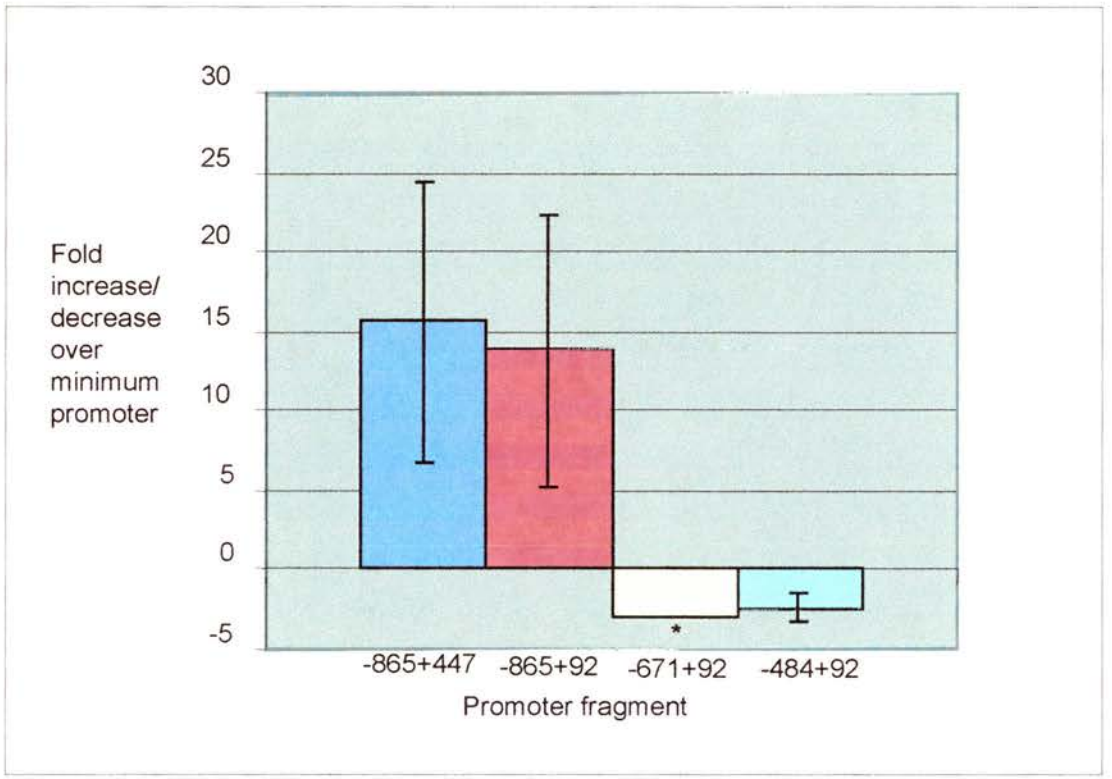

Figure 4.3: Expression of PPT-A promoter vectors in neonate DRG cultures. DRG cultures were infected with AAV vectors containing the PPT-A promoter fragments -865+447, -865+92, -671+92, -484+92 and -47+92. Cells were maintained in medium + 100 ng/ml NGF for 7 days and then harvested and assayed for luciferase activity. Results are represented as fold increase induced by promoter fragments compared to expression from the minimum promoter -47+92. Data is expressed as mean ± SEM from three to five independent experiments, whereby each experiment incorporated triplicate wells, with the exception of * which was performed only once in triplicate.

Expt	Promoter		Fold increase or decrease (D) over minimum
	-865+447	-47+92	
1	365±19.16	16.16±0.382	22.6
2	213.5±13.13	11.57±0.174	18.4
3	162.8±47.04	27.85±11.20	5.8
	-865+92	-47+92	
1	276±48.24	16.16±0.382	17.1
2	109.9±14.68	11.57±0.174	9.4
3	150.2±4.017	27.85±11.20	5.4
4	22.28±2.415	0.819±0.042	27.2
5	14.65±3.805	1.481±0.110	9.9
	-671+92	-47+92	
1	9.175±0.787	27.85±11.20	3.0 (D)
	-484+92	-47+92	
1	9.1±0.933	16.16±0.382	1.8 (D)
2	5.763±0.049	11.57±0.174	2.0 (D)
3	7.244±4.849	27.85±11.20	3.8 (D)
4	0.513±0.041	0.819±0.042	1.6 (D)
5	0.551±0.076	1.481±0.110	2.7 (D)

Table 4.4: Comparison of promoter fragment activity to minimum promoter -47+92 in neonate DRG cultures. Results from table 4.3 are represented as fold increase over minimum promoter. That is the increase in expression induced by promoter fragments -865+447, -865+92, -671+92 and -484+92 compared to expression from promoter -47+92.

4.2.2.3 Discussion

This study set out to identify the important regulatory elements within the PPT-A promoter. A series of rAAV vectors were generated that contained different PPT-A promoter fragments and were used to infect cultured DRG neurons from both adult and neonate rats. Analysis of the PPT promoter deletion fragments revealed differences between regulation of the promoter in DRGs from adult and neonate rats. These results are summarised in figures 4.4 and 4.5. In adult DRGs, the promoter fragment -865+447 directed the highest levels of luciferase reporter gene expression, followed by fragment -865+92. There is a reduction in –671+92 promoter activity when compared with -865+92 and there appears to be no apparent difference in fragments -671+92 and -484+92 promoter activities. These results suggest that the region of promoter between base pairs -865 and -671 is important for directing high levels of promoter activity and that the region between -671 and -484 does not contain enhancer elements functioning in these cultures. Additional enhancer elements important for promoter regulation may be located between -484 and -47 as removal of this sequence again results in reduced expression and the region lying between +92 and +447 may contain further enhancer elements.

In neonate cultures, the promoter fragment spanning -865+447 directed equivalent expression levels to fragment -865+92 and therefore gave no evidence of functional elements between base pairs +92 and +447. When the PPT-A flanking sequence was deleted to leave base pairs -671+92, expression levels were decreased and further 5' deletions to create fragment -484+92 did not alter expression. Together these results suggest that the region between -865 and -671 may contain elements, which can act as enhancer motifs in neonate cultures. The region spanning -671 to

-484 does not contain additional elements. This expression associated with the -484+92 promoter fragment is also less than that of the minimum promoter. This suggests the presence of potential silencer sequences in this region spanning -484 to -47 in neonates.

In conclusion, the results show that although some domains may have similar functions, expression of the PPT-A promoter may be differentially regulated between adult and neonate DRG neurons. In particular, these studies have shown the presence of potential repressor elements between -484 and -47 that is not present in adult DRG neurons. Furthermore, potential activator elements between +92 and +447 were identified in adult DRG cultures, which were not similarly observed in neonate cultures. These putative regulatory regions are summarised in figure 4.7.

It is thought that endogenous PPT-A gene expression differs between adult and neonate sensory neurons and this could be reflected in promoter expression. A number of elements could be involved in this differential expression. Examination of the nucleotide sequence of this promoter fragment reveals a number of putative transcription factor binding sites (figure 4.6) and a number of these sites have been shown to bind transcription factors (section 1.2.3, figure 4.7). In both neonate and adult DRGs the promoter spanning base pairs -865 to +92 can drive high levels of reporter gene expression and this could, in part, be attributed to the enhancer activity shown to be associated with the region spanning -865 to -671. This region is known to contain sequences that are bound by multiple double and single stranded DNA binding proteins including Sp1, AP2, hnRNPK and others (section 1.4.1). The convergence of these transcription factors to this region suggests a complex interplay between them in the regulation of the PPT-A promoter (Quinn *et al.*, 1995). AP2

expression in sensory neurons has been correlated with increased levels of PPT expression in an animal model of arthritis (Donaldson *et al.*, 1993). This previously identified AP2 site may have a role in induction of PPT-A promoter expression in adult DRG cultures in the presence of NGF.

The sequence between –671 and –484 does not appear to contain enhancer or silencer elements in either neonates or adult. This region has been shown to contain an octamer transcription factor binding site at base pairs –669 to –662 and has been shown to bind members of the family of octamer binding proteins (Mendelson *et al.*, 1998; Fiskerstrand *et al.*, 2000). Some of these proteins are found to have restricted expression within the nervous system (Rosenfeld *et al.*, 1991) and it is known that transcription factor Oct-2 is expressed in DRG neurons and is under the control of NGF (Wood *et al.*, 1992). This element has previously been shown to have enhancer function in clonal cell line NF2C as mutation of this site resulted in loss of function and was associated with loss of protein binding in EMSAs (Fiskerstrand *et al.*, 2000). The function of this 5' octamer site has not been investigated in DRGs previously and results presented here suggest that this is not acting as an enhancer in adult or neonate DRG neurons, however under certain conditions this element may play an important role in promoter inducibility. This conclusion has been drawn from the assumption that this octamer site is active in this promoter fragment. It may be the case that transcription factors binding to this site has been lost in the creation of this deletion fragment since the octamer binding site is located only 4 base pairs away from the 5' end of promoter fragment –671+92. In addition, activity of factors binding this region of the promoter may depend on them acting synergistically with elements out with this promoter fragment in order to exert an effect.

The presence of another octamer site located 3' of the transcriptional start site at base pairs + 372 to +389, has also been shown to be functionally important in the NF2C and RINm5F clonal cell lines and DRG cultures. This 3' octamer site was found to repress promoter activity (Fiskerstrand *et al.*, 1999). The results presented here suggest that the region spanning +92 to +447 may actually possess enhancer activity in adult DRG cultures. However, there are fundamental differences in the experimental procedures between these studies. Apart from the obvious method of transfection of DRG neurons, the work presented here was performed in the presence of NGF and this was not the case for the microinjection studies performed by Fiskerstrand *et al.* (1999). As stated earlier, NGF is essential for neonate DRG survival and therefore all experiments were performed in the presence of NGF so that promoter activity in adult and neonate cultures could be compared. The presence of NGF is likely to cause the induction of different transcription factors and therefore influence the results obtained. Mulderry *et al.* (1993) also found the region between +92 and +447 to possess enhancer activity. Plasmids containing promoter fragments spanning –3356 to +92 and –3356 to +447 driving the lacZ reporter gene were microinjected into adult DRG cultures and assayed for β-galactosidase activity. The latter fragment was found to be approximately 2 to 3 fold more active than the fragment -3356 to +92. In accordance with the work presented here, Mulderry *et al.* performed all experiments in the presence of NGF. NGF can activate both PPT-A expression and a variety of transcription factors. These include AP1 and cAMP responsive element binding protein (CREB), the octamer binding proteins and the basic helix-loop-helix proteins (bHLH) (section 1.2.2.). In addition to the octamer binding site, this region spanning +92 to +447 has also been shown to contain a

potential binding site (termed E box motif) for the bHLH family of transcription factors (section 1.2.2). Together these elements may be important for this observed induction in promoter activity in adult DRG neurons.

The region between –484 and –47 may contain transcriptional regulatory motifs that can function as enhancer elements in adult DRG cultures. In neonate DRG neurons there is some evidence for these sequences being moderate repressors of promoter activity. This region contains several AP1-CRE-like elements and E box motifs. The functional consequence of binding to an AP1 site can be either enhancement or repression of transcription (Quinn *et al.*, 1989; Takimoto *et al.*, 1989). AP1- and CRE-like elements have been shown to function synergistically with other promoter elements to work as a tissue specific enhancer or repressor (Chu *et al.*, 1991; Hyman *et al.*, 1989; Leonard *et al.*, 1992; Zhang and Young, 1991). The AP1 transcription factor complex consists of dimeric protein complexes composed of members of the c-jun and c-fos family of molecules. Members of the AP1 family of transcription factors can also dimerise with members of the cAMP responsive element binding proteins to bind both AP1 and CRE consensus sequences (section 1.2.2.2). AP1 and CRE elements often act synergistically to regulate gene expression. These elements could therefore act as potential *cis*-regulatory elements through which a vast range of stimuli could act and these elements could contribute to the differences in promoter activity seen in adult and neonate sensory neurons. Growth factors, inflammation, steroids and cocaine have been shown to regulate expression of these proteins directly (Bartel *et al.*, 1989; Hope *et al.*, 1992; Moratalla *et al.*, 1993, Quinn, 1991; Spiro *et al.*, 1993). Similarly the E motifs located in this region may also contribute to enhancer or repressor activity. As stated above, E box

elements are recognised and bound by the bHLH family of proteins. Like members of the AP1 and CRE families, these bHLH proteins form homo- or heterodimer complexes with certain other members of the family (section 1.2.2.1). Specific bHLH factors are expressed by neurons in addition to constitutive factors (Johnson *et al.*, 1992) and are also involved in tissue specific gene expression (Ball *et al.*, 1992; Murre *et al.*, 1989a; Therrien and Drouin, 1993).

There are many potential factors, which can act to mediate expression of the PPT promoter. Functional determination of important transcriptional regulators is a complex process. The work described here has contributed to the delineation of potential regions of the PPT-A promoter that are important for regulation of PPT-A expression. It has provided the basis for future studies to define the specific elements that may be involved in basal or inducible PPT-A promoter activity.

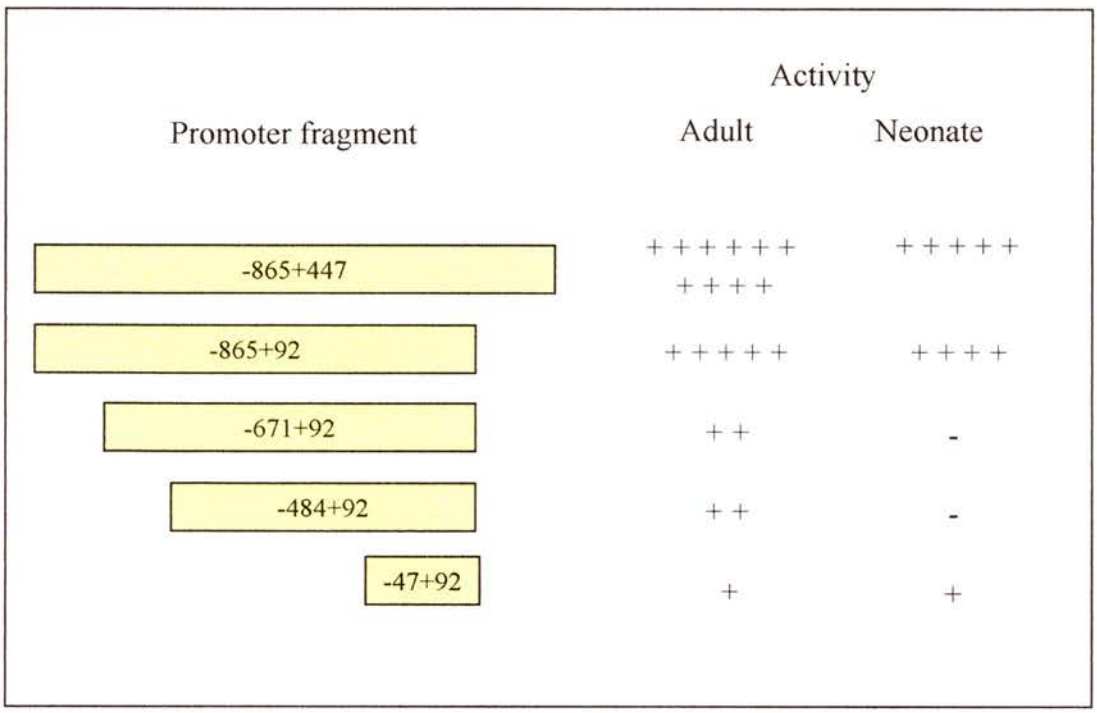

Figure 4.4: Summary of promoter results in cultured DRGs from neonate and adult DRG. Figure shows promoter fragments and the resulting activity represented by +and -. See text for details.

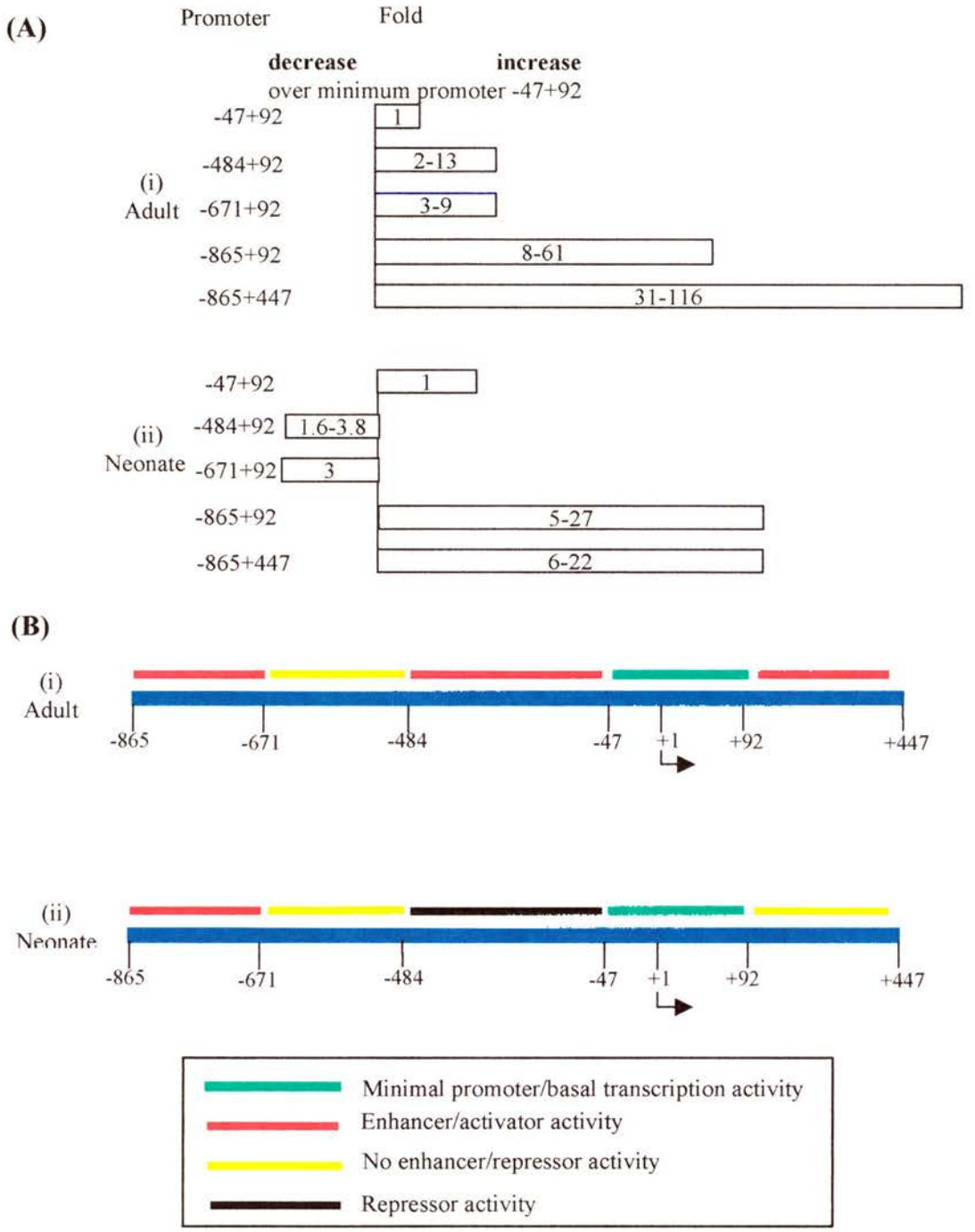

Figure 4.5: Differential regulation of the PPT-A promoter in cultured adult and neonate DRGs. Figure shows (A) the fold increase or decrease of promoter fragment expression over the minimum promoter in both adult (i) and neonate (ii) DRGs. These results reveal the differences in promoter activity in adult and neonates and the (B) possibility that different enhancer and repressor elements are utilised for transcription of the PPT-A promoter in adult (i) and neonate (ii) cultured DRGs.

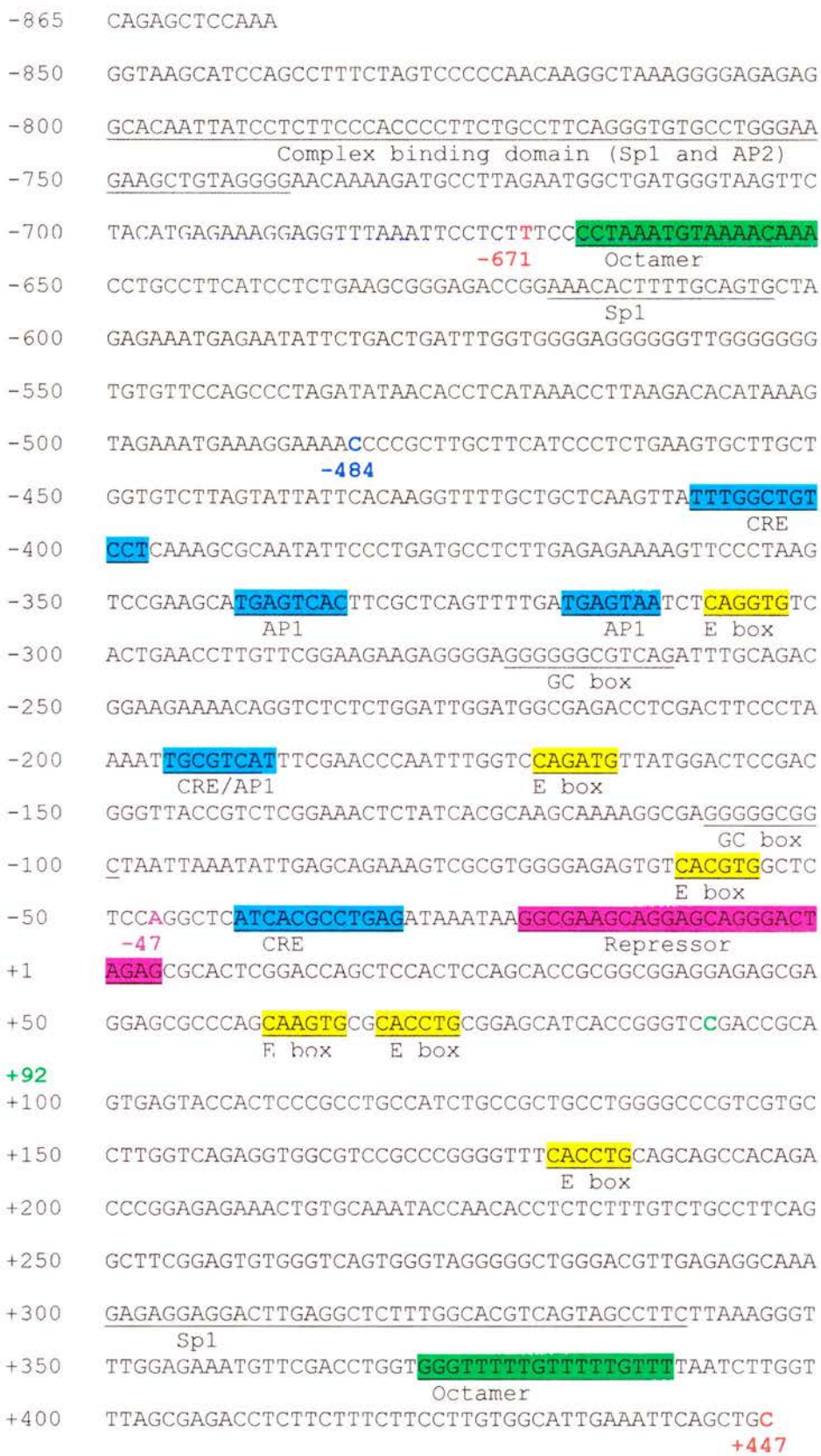

Figure 4.6: Sequence of promoter fragment spanning –865+447 base pairs. Nucleotides also -671, -484, -47 and +92 highlighted to indicate where sequence of each promoter fragment would start and finish. The positions of transcription factor binding sites are shown.

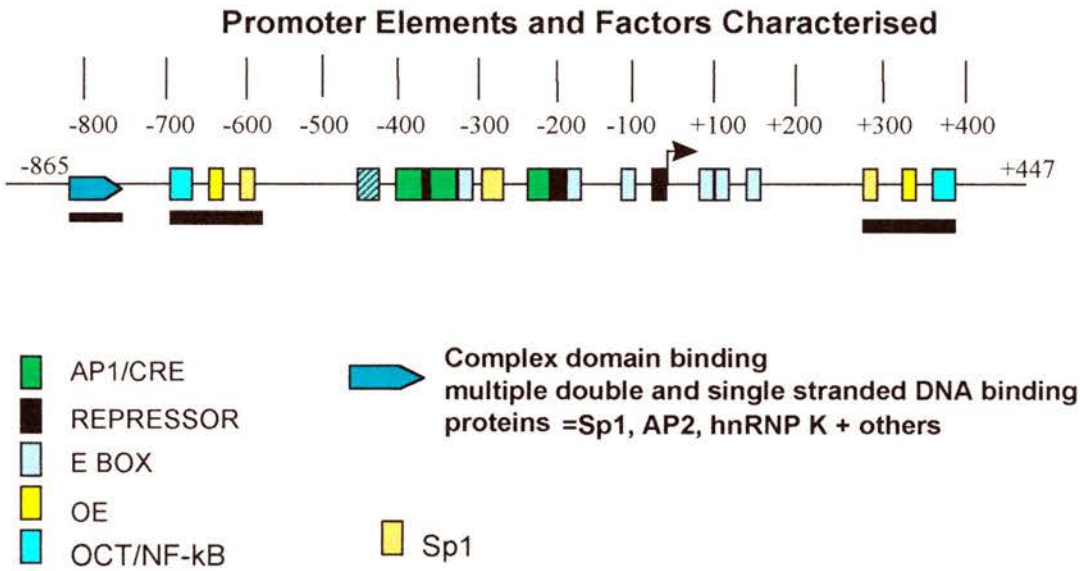

Figure 4.7: Diagram of transcription factor interactions with the PPT-A promoter.

4.3 Regulation of PPT-A promoter activity: E box mutant studies

4.3.1 Background

This study set out to investigate the role of the 'E box motif' located between –67 and –47 base pairs of the proximal PPT-A promoter. Previous work addressing the function of this site has found that it may be important for the transcriptional regulation of the PPT-A promoter. When this sequence is linked to a heterologous promoter it has been shown to drive high levels of the reporter gene expression in PC12 and HeLa cell lines (Mendelson *et al.*, 1995) and in DRG neurons (Paterson *et al.*, 1995a). NGF induced the activity of this same reporter gene construct in transfected PC12 cells but this induction of expression was not observed in microinjected DRG neurons (Paterson *et al.*, 1995a). The activity of the PPT promoter fragment –865+92 in PC12 cells was compared to a mutated version of this same promoter fragment, in which the –60 E box site was disrupted. Following forskolin-potassium evoked depolarisation the mutant construct showed much lower reporter gene expression compared to the wild-type promoter. Under basal conditions, the PPT-A promoter is inactive in PC12 cells, however the combined stimuli of forskolin and depolarisation, is necessary to achieve expression of the PPT-A promoter. Depolarisation induces members of the AP1 family of transcription factors (Bartel *et al.*, 1989) and forskolin induces members of the cAMP response element binding (CREB) family of transcription factors (Montminy *et al.*, 1986). E box motifs are recognised by the basic helix-loop-helix family (bHLH) of transcription factors, specifically members Max and USF (Paterson *et al.*, 1995b). Members of these three families of transcription factors are known to heterodimerise with other family members to allow inducible and tissue-specific gene expression

and can be induced in response to NGF (section 1.2.2.1). It is possible this element may be important for the regulation of the PPT promoter and may have role in mediating the response to NGF and other growth factors.

The activity of this -60 mutant promoter fragment has not yet been investigated in DRG neurons. The activities of the promoter fragments –865+92 and -60 mutant were compared together with the minimum promoter fragment –47+92 in DRG neurons that were maintained it the presence or absence of NGF. This was to determine if this site is important for regulating the proximal promoter in DRG neurons and if it has a role in the NGF response associated with this proximal promoter.

4.3.2 Results

To address the role of the E box binding motif spanning base pairs –47 to –67 of the rPPT-A promoter, recombinant AAV vectors were generated that contained a mutant version of the PPT promoter fragment –865+92, driving the luciferase reporter gene (AAV-60mut-LUC) (section 2.2.3.2). The mutant promoter fragment termed –60mut contained the insertion of a 10 bp oligonucleotide at nucleotide –60, and has been previously described to disrupt PPT-A promoter activity (Paterson *et al.*, 1995a; 1995b). This was compared with the vectors containing the wild-type promoter fragment –865+92 (AAV-865+92-LUC) and the minimum PPT promoter fragment spanning base pairs –47+92 (AAV-47+92-LUC). These rAAV particles, AAV-865+92-LUC, AAV-60mut-LUC and AAV-47+92-LUC, were used to infect DRG neuronal cultures from adult rats and were subsequently maintained in the presence or absence of 100 ng/ml mouse recombinant 7S NGF (section 2.2.10.1). 7

days following infection, DRG cultures were harvested, assayed for luciferase activity and the activity of each promoter compared (section 2.2.10.3). Each experiment was carried out four times each time in triplicate.

It was found that the promoter activity of fragment –865+92 was equal to that of the –60mut promoter fragment in cultures which had been maintained in NGF for the duration of the infection period (table 4.5). The activity of both these fragments being approximately 4 to 11 times greater than that of the minimum promoter (table 4.5).

In the absence of NGF it was found that the activity of fragment –60mut was approximately 8 fold greater than that of fragment –865+92 and approximately 16 to 47 times greater than the activity of the minimum promoter (table 4.6). When the promoter activity of fragment –865+92 was compared to the minimal promoter in the absence of NGF it is only 2.6 to 9 times greater than –47+92 promoter activity (table 4.6). These results, summarised in figures 4.8 and 4.9 suggest that disruption of this E box motif at –60 base pairs resulted in a higher level of promoter activity in DRG cells in the absence of NGF when compared to the minimum promoter activity.

To determine whether the –60mut promoter activity was decreased in the presence of NGF, cultured DRG neurons were infected with AAV-60mut-LUC and maintained in the presence or absence of NGF. In a separate study, cultured DRG neurons were infected with AAV -865+92 and also maintained in the presence or absence of NGF. 7 days post-infection, cells were lysed and cell extracts assayed for luciferase activity. Table 4.7 shows that in the presence of NGF, luciferase values of those cells infected with AAV-60mut-LUC are lower than in the absence of NGF and therefore promoter activity is slightly repressed (ranging from 1.5 to 4 fold). In

contrast, in the presence of NGF the activity of AAV-865+92-LUC is increased (ranging from 2.5 to 7.8 fold) compared to untreated samples (table 4.8).

Expt	Luciferase activity			Fold incr.	
	-865+92	-60mut	-47+92	-865+92 over min	-60mut over min
1	0.952 ± 0.137	0.744 ± 0.188	0.152 ± 0.013	6.3	4.9
2	0.403 ± 0.151	0.348 ± 0.090	0.035 ± 0.004	11.5	9.9
3	10.54 ± 2.726	20.03 ± 0.976	1.85 ± 0.461	5.7	10.8
4	2.899 ± 1.202	2.394 ± 0.548	0.631 ± 0.071	4.6	3.8

Table 4.5: Comparison of promoter fragments –865+92, -60mut and –47+92 in the **presence of NGF**. Adult DRG cultures were infected AAV vectors containing the PPT promoters –865+92, -60mut and –47+92 (minimal promoter). Cells were maintained in medium + NGF for 7 days and then harvested and assayed for luciferase activity. The luciferase values are represented as mean and std. error of triplicate wells for each sample. Fold increase represents the increase in expression induced by fragments –865+92 and –60mut compared to expression from minimal promoter –47+92.

Expt	Luciferase activity			Fold incr.	
	-865+92	-60mut	-47+92	-865+92 over min	-60mut over min
1	2.265 ± 0.732	16.75 ± 2.129	0.852 ± 0.202	2.7	19.7
2	4.941 ± 1.114	25.80 ± 3.524	0.550 ± 0.034	9.0	46.9
3	3.845 ± 1.193	28.14 ± 6.572	-	-	-
4	1.298 ± 0.783	8.297 ± 2.866	0.508 ± 0.186	2.6	16.3

Table 4.6: Comparison of promoter fragments –865+92, -60mut and –47+92 in the **absence of NGF.** Adult DRG cultures were infected AAV vectors containing the PPT promoters –865+92, -60mut and –47+92 (minimal promoter). Cells were maintained in medium only for 7 days and then harvested and assayed for luciferase activity. The luciferase values are represented as mean and std. error of triplicate wells for each sample. Fold increase represents the increase in expression induced by fragments –865+92 and –60mut compared to expression from minimal promoter –47+92.

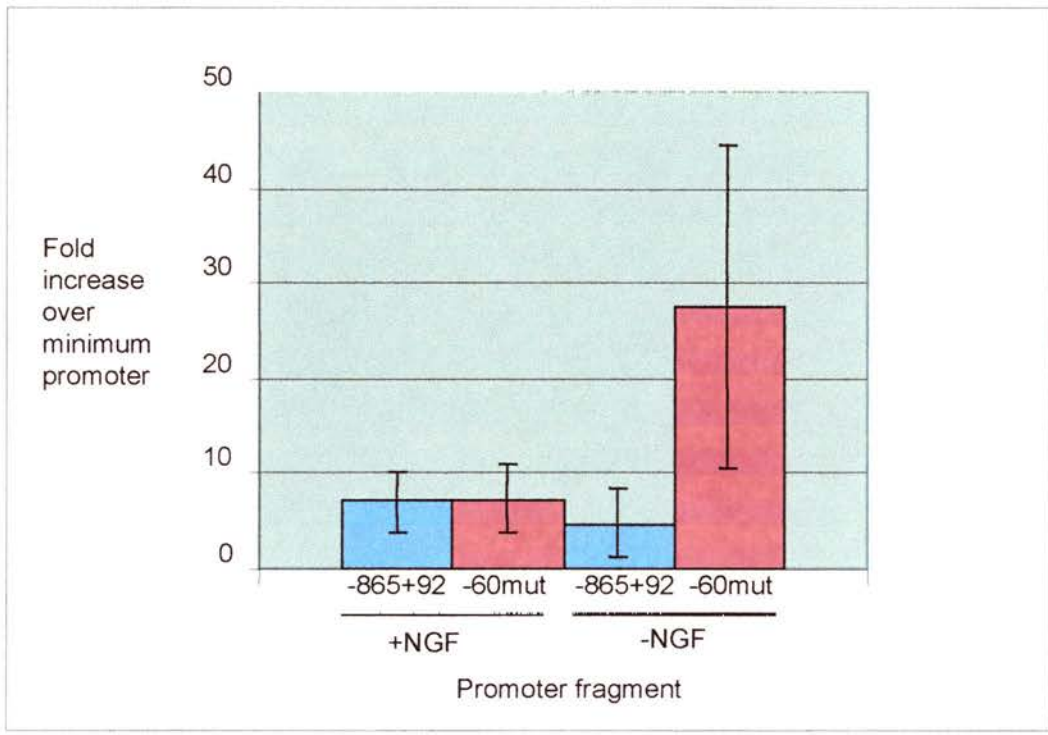

Figure 4.8: Comparison of promoter PPT-A promoter fragments -865+92 and -60mut in the presence or absence of NGF. DRG cultures were infected with AAV vectors containing the PPT-A promoter fragments -865+92, -60mut and -47+92. Cells were maintained in medium +/- 100 ng/ml NGF for 7 days and then harvested and assayed for luciferase activity. Results are represented as fold increase induced by promoter fragments compared to expression from the minimum promoter -47+92. Data is expressed as mean ± SEM from three or four independent experiments, whereby each experiment incorporated triplicate wells.

Expt	Luciferase activity –60mut		Fold decr. NGF treated over untreated
	- NGF	+NGF	
1	1.270	0.348	3.6
2	0.718	0.418	1.7
3	0.089	0.047	1.9
4	42.81	27.99	1.5
5	17.85	6.288	2.8

Table 4.7: Promoter activity of –60mut in the presence or absence of NGF. Adult DRG cultures were infected with AAV vectors containing the PPT promoter –60mut driving the luciferase reporter gene and maintained in the presence or absence of 100 ng/ml NGF. Cells were harvested and assayed for luciferase activity 7 days post-infection. Luciferase values are given as mean and std. error of triplicate wells for each sample. Fold decrease represents the reduction in expression induced by NGF treatment compared to untreated samples.

Expt	Luciferase activity –865+92		Fold incr. NGF treated over untreated
	- NGF	+ NGF	
1	0.015 ± 0.010	0.066 ± 0.019	4.4
2	2.975 ± 0.019	7.463 ± 1.791	2.5
3	6.245 ± 1.165	46.68 ± 2.032	7.8
4	10.93 ± 5.979	51.08 ± 13.56	4.7

Table 4.8: Promoter activity of –865+92 in the presence or absence of NGF. Adult DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained in the presence or absence of 100 ng/ml NGF. Cells were harvested and assayed for luciferase activity 7 days post-infection. Luciferase values are given as mean and std. error of triplicate wells for each sample. Fold increase represents the increase in expression induced by the addition of NGF compared to untreated samples.

4.3.3 Discussion

The aim of this study was to investigate the function of one of the E boxes in the PPT-A promoter in DRG neurons. Adult DRG neurons were infected with AAV vectors containing the PPT-A promoter -865+92 and a mutant version of this promoter termed -60mut. This mutant contained a disruption in the E box motif, which spans base pairs -47 to -67 of the PPT promoter. The activities of these constructs were compared both in neuronal cultures that were maintained in medium only and those that were maintained in medium containing NGF. It was found that when NGF is absent, the -60mut construct has greater activity than that of -865+92 (table 4.5). Following NGF treatment, -865+92 and -60mut promoter fragments possessed equal activities (table 4.6). Further experiments examined the effect of NGF on each of the promoter fragments (table 4.7 and 4.8). The activity of the -60mut fragment was greater in the absence of NGF than in the presence. The opposite effect was observed for -865+92 promoter activity, whereby promoter activity was increased in the presence of NGF.

These results confirm that this E box site may play an important role in the regulation of the PPT-A proximal promoter. It has been previously suggested that this site might be involved in NGF induction of the PPT-A gene. The results presented here indicate this E box motif may have a regulatory role that is associated with the basal levels of promoter expression. It can be hypothesised that in DRG neurons in the absence of NGF, the PPT-A proximal promoter activity is normally repressed by the presence of factors binding this E box motif (figure 4.10). These studies provide no clear evidence to support a role for this E box motif in the NGF induction of the PPT-A promoter. In the presence of NGF, the fold increase over the

minimum promoter is 3.8 to 10.8 and in the absence of NGF, it is 16.3 to 49.9, therefore the -60 mutant fragment is more active in the absence of NGF than in the presence. In addition, when the -60mut fragment was compared in the presence or absence of NGF, untreated samples did have greater luciferase activity than those that were NGF treated. It can be hypothesised that NGF might mediate its effect by relieving repression of this E box site that was observed under basal conditions. If this is the case, induction by NGF of this –60mut promoter may not have been observed under these experimental conditions since the creation of this mutation has relieved repression under both basal and NGF treated conditions and therefore may mask the normal effect of NGF. Similarly it can be hypothesised that NGF may mediate its effect by activating transcription factors that bind elements on the PPT-A promoter other than this E box motif. Further experiments would need to be performed before any conclusions could be drawn regarding the role of this E box in NGF induction of the PPT-A promoter (figure 4.10).

Previous work has shown that this E box site can function as a transcriptional activator in microinjected DRG neurons and PC12 cells (Paterson *et al.*, 1995a; 1995b). This work by Paterson *et al.* looked at the E box activity in DRG neurons in isolation. In addition activity of the disrupted E box in context of entire promoter was examined in PC12 cells, following forskolin and potassium depolarisation. The use of different cell types and induction conditions are bound to influence the precise repertoire of transcription factors present and this is likely to modulate the function of the PPT-A E box. As previously stated E box motifs are bound by the bHLH family of transcription factors. These can form homo- or hetero-dimers with different member of the bHLH family and with members of other transcription factor families

(section 1.2.2.1). Furthermore, the specificity of binding to the E box may involve recognition of flanking sequences in addition to the core binding motif therefore looking at the E box fragment in isolation may give different results to those generated in the context of the entire promoter. Therefore the flexibility of interaction between transcription factors may facilitate whether this E box motif is to function as an activator or repressor, facilitating tissue-specific and inducible PPT-A gene expression.

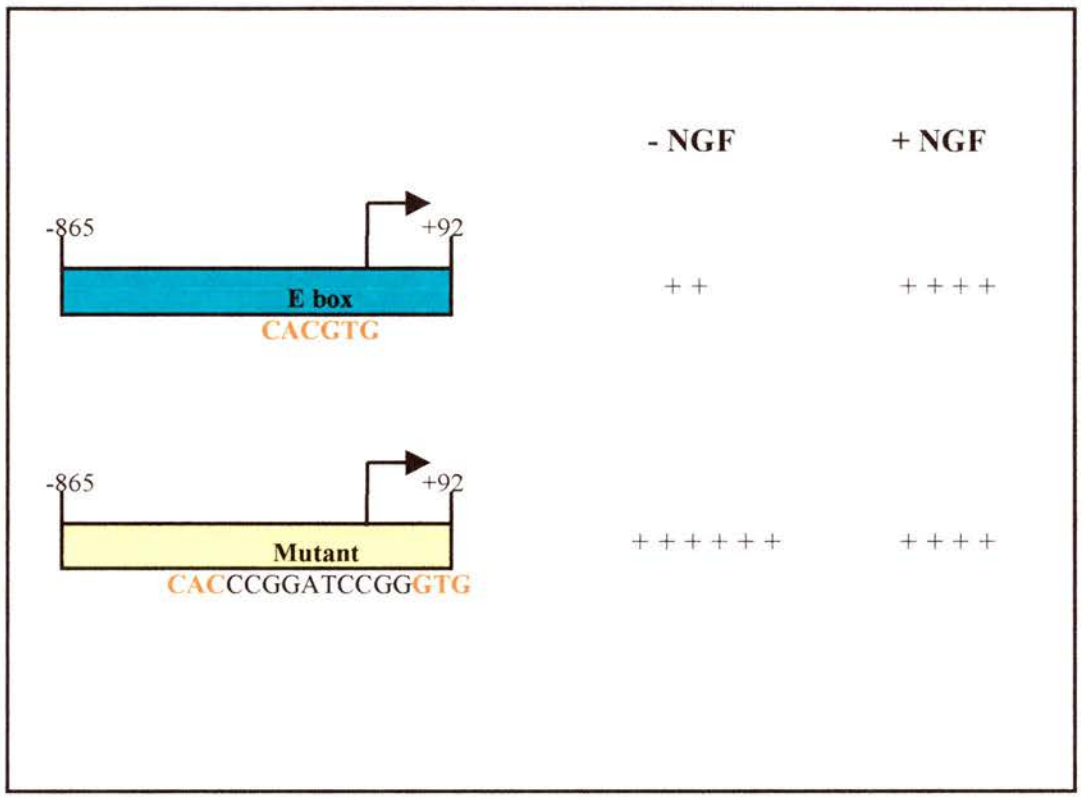

Figure 4. 9: Summary of PPT-A promoter -865+92 and mutant version -60mut activity in the presence or absence of NGF. Figure shows the E box sequence in the wild-type promoter fragment (red) and the mutant version (original sequence shown in red and 10 bp oligonucleotide is shown in black).

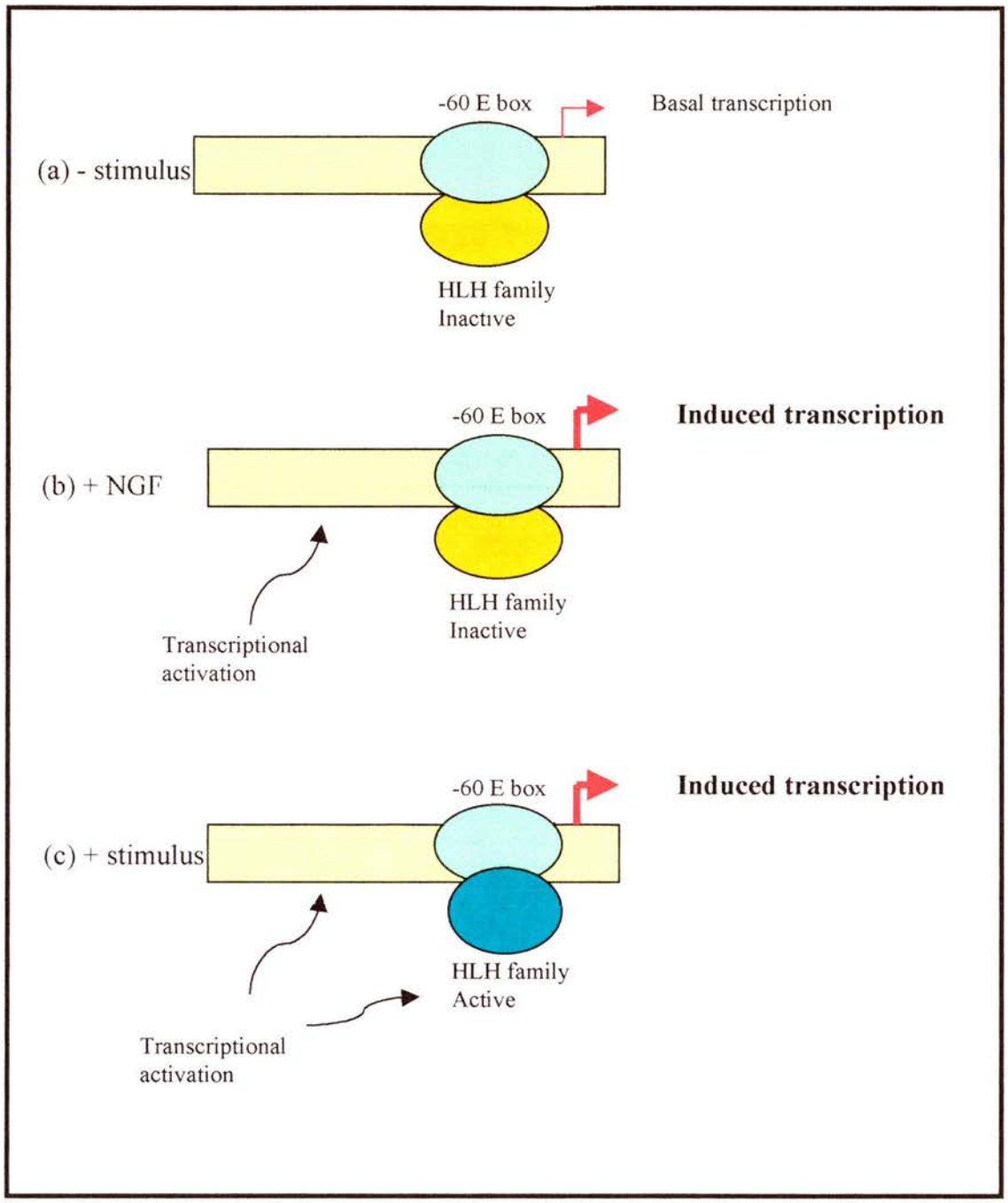

Figure 4.10: Hypothesis for the role of E box motif in regulation of the PPT-A promoter. It is thought that the -60 E box motif is repressed under normal conditions (a). It is unclear whether it is involved in the induction of PPT-A promoter activity by NGF treatment. NGF induction may be due to the activation of transcription factors acting on promoter elements other than the E box motif (b). It is possible that this E box site is activated in response to NGF and other stimuli (c).

4.4 Effect of Growth factors on the PPT-A proximal promoter

4.4.1 Background

The aim of this study was to determine if the PPT-A proximal promoter is responsive to a variety of growth factors in cultured DRG neurons. The PPT gene is regulated directly and indirectly by a variety of stimuli including steroids, cocaine, inflammation and peripheral axotomy (section 1.1.3). In addition, members of the neurotrophin family and other growth factors are thought to influence regulation of the PPT-A gene. Little is known about the effect of neurotrophic factors other than nerve growth factor (NGF) on PPT-A gene expression in DRG neurons. NGF can regulate PPT mRNA levels in DRG neurons in culture and *in vivo* (section 1.1.3). However, other growth factors and members of the NGF-related neurotrophin family have also been implicated in regulating SP expression in sensory neurons (Mulderry, 1994). If these factors play a role in influencing neuropeptide levels in DRG neurons, it is possible that regulation is at the level of transcription of the PPT-A gene. Sequence analysis of the PPT-A gene showed the presence of many putative transcription factor binding sites which may have a role in mediating the effects of these stimuli (section 1.2.2). Furthermore, DNA binding studies showed the majority of important regulatory elements reside in the proximal promoter region spanning nucleotides -865 to +447 (section 1.2.3). This PPT-A promoter fragment has previously been shown to be NGF responsive in cultured DRG neurons when delivered as a rAAV particle and therefore must contain regulatory elements that can mediate the effect of NGF (Harrison *et al.*, 1999). It was thought that this promoter fragment –865+92 may contain regulatory elements that can act to mediate the effect of other growth factors. The effects of growth factors nerve growth factor (NGF),

brain derived neurotrophic (BDNF) and glial cell-derived neurotrophic factor (GDNF), together with cytokines leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), on PPT-A promoter activity was examined. These factors were chosen because they have all been implicated in regulating neuronal phenotypes and have been associated with distinct populations of DRG neurons.

NGF and BDNF are both members of the neurotrophin family of growth factors (section 1.3.1). Although adult sensory neurons do not require neurotrophins for their continued survival, the neurotrophins are required for the continued maintenance of particular mature phenotypic characteristics, including peptide content and physiologic responsiveness. Neurotrophins also play a role in the regulation of neuronal growth, and particularly in growth or regeneration as a response to injury (Segal and Greenberg, 1996). NGF is known to have an important role in adult sensory neuron function, particularly nociception (Lewin and Mendell, 1993). BDNF is also required for neuronal plasticity and is thought to have nociceptive function distinct from that of NGF in adult DRG neurons (Thompson *et al.*, 1999; Kerr *et al.*, 1999; Shu and Mendell, 1999).

In addition to the neurotrophins, several other proteins are thought to influence phenotypic characteristics. LIF and IL-6 are members of the neuropoietic cytokine family. These cytokines have been shown to influence the survival and differentiation of various neuronal subpopulations in the central and peripheral nervous system (Fann and Patterson, 1994; Sendtner *et al., 1994;* Stahl and Yancopoulos, 1994; Pennica *et al.*, 1995; Horton *et al.*, 1998). LIF and IL-6 may play a regulatory role in the interaction between the nervous and immune systems and both LIF and IL-6 are thought to be involved in altering neuropeptide phenotype

in adult sensory neurons following nerve injury (Corness *et al.*, 1996; Sun and Zigmond, 1996a; 1996b; Thompson *et al.*, 1998).

GDNF is member of the transforming growth factor β (TGFβ) superfamily and is known to play an important role in the development and survival of dopaminergic neurons (Poulsen *et al.*, 1994; Beck *et al.*, 1995; Bowankamp *et al.*, 1995). GDNF has been shown to be associated with sensory neurons (Holstege *et al.*, 1998; Bennet *et al.*, 1998b; Kashiba *et al.*, 1998) and induce SP expression in DRG cultures (Adler, 1998).

4.4.2 Results

To determine if these growth factors can directly regulate the PPT-A promoter, DRG were dissected from adult rats and the neurons cultured (section 2.2.7.1). In the initial stages of producing these cultured DRGs, cells were plated out in medium containing NGF to promote neurite outgrowth. Routinely after 24-48 hours, medim containing exogenous NGF was removed and cells were maintained in medium containing no growth factors for at least 24 hours (section 2.2.10.2). AAV vectors that contained the PPT-A promoter spanning base pairs -865+92 driving the luciferase reporter gene (termed AAV-865+92-LUC) was used to infect DRG neurons (section 2.2.10.1). The infected neurons were subsequently maintained in the presence or absence of each particular growth factor for 7 days. DRG cultures were harvested and assayed for luciferase reporter gene product activity (section 2.2.10.3). It was important in these experiments to ensure that results obtained are due to the growth factor acting specifically on the promoter construct and not due to variations in neuronal survival. Adult DRGs have been reported to survive in culture

independently of added neurotrophic factors (Lindsay, 1988) therefore the effect of trophic factors on promoter activity should be uncomplicated by cell survival effects. However, to eliminate any possible effect on survival, the effect of growth factors on the CMV promoter was also examined. This also eliminates the possibility that any response observed is due to artefacts associated with virus genome structure. Accordingly DRGs were also separately infected with AAV vectors containing the CMV promoter driving the luciferase reporter gene (termed AAV-CMV-LUC). This process is shown in figure 4.11.

Another important aspect associated with these experiments is that there is a high degree of variability in promoter activity both between and sometimes within experiments. In addition, only a small number of samples are available for analysis in each experiment and a relatively small number of cells make up each sample. These factors together make variation appear large and also mean that statistical or quantitative analysis cannot be performed. However, trends in induction or repression of promoter activity can be investigated and reveal important information about the regulation of the PPT-A promoter. Due to these constraints it was decided that if a growth factor resulted in a 2 fold or greater increase or decrease in luciferase activity, it could be described as having an effect on the PPT-A promoter. In addition the concentrations of exogenous growth factor employed by these experiments were similar to those that have been used in the literature to induce endogenous PPT-A gene expression (NGF: Lindsay and Harmer, 1989; GDNF: Adler, 1998; CNTF, BDNF, LIF: Mulderry, 1994; IL-6: Fann and Patterson, 1994).

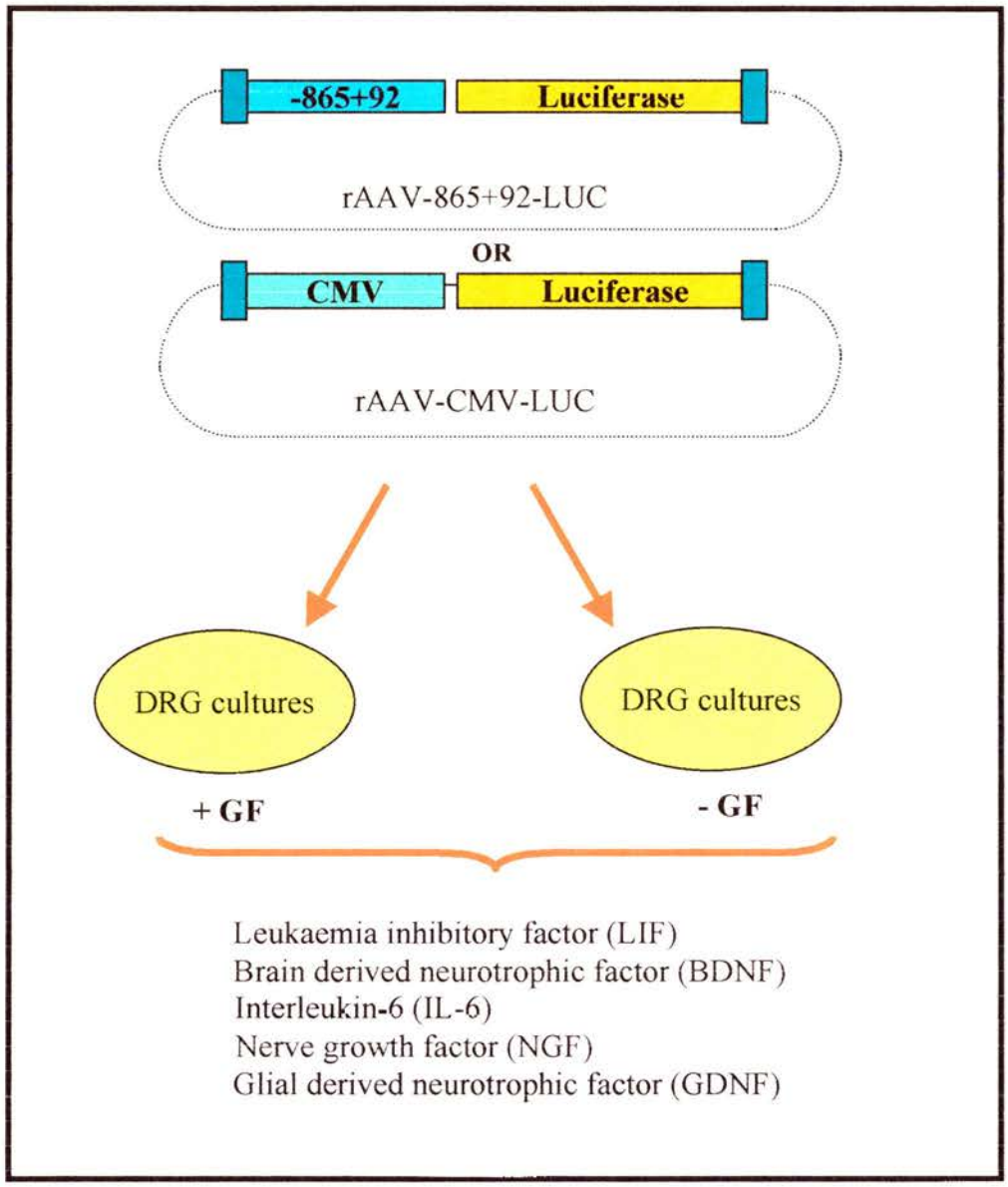

Figure 4.11: Stimulus inducibility of the PPT-A promoter: Summary of growth factor experiments. Cultured DRG were infected with AAV vectors containing the PPT-A proximal promoter driving the luciferase reporter gene (AAV-865+92-LUC). Infected cultures were subsequently maintained in medium +/- growth factor (GF). Separate DRG cultures were also infected with AAV vectors containing the CMV promoter driving luciferase (AAV-CMV-LUC). This ensured any effect observed was due to GFs acting specifically on promoter. The effects of a variety of growth factors were studied.

4.4.2.1 Effect of NGF

The effect of 100 ng/ml of exogenous mouse recombinant 7S NGF on the PPT-A promoter was investigated. Table 4.9 shows the mean values of each experiment when AAV-865+92-LUC infected DRG cultures were treated with NGF or untreated. This experiment was performed four times, each time in triplicate. In each experiment, NGF treated cells displayed increased luciferase values compared to cells in which exogenous NGF was absent, suggesting the induction of PPT promoter activity by NGF. Induction ranges from 2.5 fold to 7.8 fold. NGF did not influence levels of luciferase activity in those cells infected with AAV-CMV-LUC (table 4.14).

4.4.2.2 Effect of GDNF

The effect of 25 ng/ml rat recombinant GDNF on the PPT-A promoter was examined four times in triplicate. Table 4.10 shows the mean values of each experiment. Cultures infected with AAV-865+92-LUC and maintained in GDNF showed greater luciferase values. This increase ranged from 2.4 to 4.4 fold compared to cells, which were not treated with GDNF, implying GDNF may be acting to induce PPT promoter activity. When the same experiment was performed on cells infected with AAV-CMV-LUC, GDNF did not affect luciferase values (table 4.14)

4.4.2.3 Effect of BDNF

The effect of 50 ng/ml human recombinant BDNF on the PPT-A –865+92 promoter fragment in DRG cultures was examined in four separate experiments (table 4.11). It was found that in those cells treated with BDNF, luciferase values

were 1.6 to 2.7 times greater than those cells that in which exogenous BDNF was absent. It is unclear if BDNF is acting to significantly induce promoter activity.

4.4.2.4 Effect of LIF and LIF+NGF

To examine the effect of LIF alone and NGF together with LIF, infected neuronal cultures were maintained in medium containing no growth factor, medium containing LIF alone and medium containing LIF and NGF together. This experiment was performed six times each in triplicate (table 4.12). When cells were maintained in the presence of 25 ng/ml mouse recombinant LIF only luciferase values were reduced by 2 to 4 fold compared to cells in which LIF was absent. When cells were maintained in both 25 ng/ml LIF and 100 ng/ml mouse recombinant 7S NGF, the results were much more variable. In experiments 2 and 6, the addition of NGF and LIF together showed increased luciferase activity (3.5 and 3 fold) compared to untreated. However, in experiments 3 and 4, a clear reduction in luciferase activity was observed (2.6 to 5.4 fold) and in experiments 1 and 5, no real effect on luciferase values was observed when NGF and LIF were added. These results suggest that LIF can act to repress PPT promoter activity, however it is unclear what action NGF and LIF are having on the promoter when provided together.

4.4.2.5 Effect of IL-6

The effect of IL-6 on PPT-A promoter activity was examined three times, each in quadruplicate. Cultures infected with AAV-865+92-LUC were maintained in the presence or absence of 25 ng/ml mouse recombinant IL-6 and the mean luciferase

values obtained for each experiment are shown in table 4.13. In experiments 1 and 2, IL-6 does not appear to be influencing luciferase activity, however in experiment 3 there is a 6 fold increase in luciferase activity in IL-6 treated samples compared to cells maintained in the absence of IL-6. Again, it is unclear if IL-6 is significantly acting to influence the PPT-A promoter.

Experiment	Luciferase values		
	- NGF	+ NGF	Fold incr. NGF treated over untreated
1	0.015 ± 0.010	0.066 ± 0.019	4.4
2	2.975 ± 0.019	7.463 ± 1.791	2.5
3	6.245 ± 1.165	46.68 ± 2.032	7.8
4	10.93 ± 5.979	51.08 ± 13.56	4.7

Table 4.9: Effect of NGF on the PPT-A promoter. Adult DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained in the presence or absence of 100 ng/ml NGF. Cells were harvested and assayed for luciferase activity 7 days post-infection. Luciferase values are given as mean and std. error of triplicate wells for each sample. Fold increase represents the increase in expression induced by the addition of NGF compared to cells that were maintained in the absence of NGF (untreated).

Experiment	Luciferase values		
	- GDNF	+ GDNF	Fold incr. GDNF treated over untreated
1	33.0 ± 14.43	144.3 ± 51.09	4.4
2	16.82 ± 7.03	64.47 ± 6.31	3.8
3	2.61 ± 0.895	6.533 ± 1.161	2.5
4	0.685 ± 0.163	2.357 ± 1.010	3.4

Table 4.10: Effect of GDNF on the PPT-A promoter. Adult DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained in the presence or absence of 25 ng/ml GDNF. Cells were harvested and assayed for luciferase activity 7 days post-infection. Units of expressed luciferase are given as mean and std. error of triplicate or quadruplicate wells for each sample. Fold increase represents the increase in expression induced by the addition of GDNF compared to cells that were maintained in the absence of GNDF (untreated).

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Experiment	Luciferase values		
	- BDNF	+ BDNF	Fold incr. BDNF treated over untreated
1	0.396 ± 0.051	0.929 ± 0.405	2.3
2	1.818 ± 0.042	3.103 ± 0.520	1.7
3	0.781 ± 0.133	1.263 ± 0.108	1.6
4	0.048 ± 0.003	0.135 ± 0.023	2.8

Table 4.11: Effect of BDNF on the PPT-A promoter. Adult DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained in the presence or absence of 25 ng/ml BDNF. Cells were harvested and assayed for luciferase activity 7 days post-infection. Units of expressed luciferase are given as mean and std. error of triplicate or quadruplicate wells for each sample. Fold increase represents the increase in expression induced by the addition of BDNF compared to cells that were maintained in the absence of BDNF (untreated).

Experiment	Luciferase values				
	- GF	+ LIF	+ LIF + NGF	Fold decr. LIF treated over untreated	Fold incr. (I) or decr. (D) LIF + NGF treated over untreated
1	34.11 ± 1.010	17.13 ± 2.586	52.12 ± 20.24	2.0	1.5 (I)
2	2.553 ± 0.855	-	9.522 ± 0.878	-	3.5 (I)
3	5.594 ± 2.913	1.41 ± 0.908	1.027 ± 0.277	4.0	5.5 (D)
4	4.898 ± 2.718	1.543 ± 0.431	1.881 ± 0.303	3.2	2.6 (D)
5	0.166 ± 0.052	0.086 ± 0.014	0.151 ± 0.041	2.0	1.1 (D)
6	0.535 ± 0.055	0.262 ± 0.064	1.600 ± 0.576	2.0	3.0 (I)

Table 4.12: Effect of LIF and NGF together and LIF alone on the PPT-A promoter. Adult DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained in the presence or absence of 25 ng/ml LIF or 25 ng/ml LIF + 100 ng/ml NGF. Cells were harvested and assayed for luciferase activity 7 days post-infection. Units of expressed luciferase are given as mean and std. error of triplicate or quadruplicate wells for each sample. Fold increase or decrease represents the increase or decrease in expression induced by the addition of LIF and NGF compared to cells that were maintained in the absence of NGF and LIF (untreated).

Experiment	Luciferase values		
	- IL-6	+ IL-6	Fold incr. IL-6 treated over untreated
1	54.27 ± 18.81	88.39 ± 12.64	1.6
2	4.467 ± 1.456	5.051 ± 1.359	1.1
3	2.021 ± 0.304	12.10 ± 5.036	6.0

Table 4.13: Effect of IL-6 on the PPT-A promoter. Adult DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained in the presence or absence of 25 ng/ml IL-6. Cells were harvested and assayed for luciferase activity 7 days post-infection. Units of expressed luciferase are given as mean and std. error of triplicate or quadruplicate wells for each sample. Fold increase represents the increase in expression induced by the addition of IL-6 compared to cells that were maintained in the absence of IL-6 (untreated).

Growth factor	Experiment	Luciferase values		
		- GF	+ GF	Fold incr. (I) or decr. (D) GF treated over untreated
NGF (100 ng/ml)	1	0.294 ± 0.024	0.276 ± 0.043	0.9 (D)
	2	0.394 ± 0.038	0.560 ± 0.051	1.4 (I)
LIF (25 ng/ml)	1	0.121 ± 0.006	0.165 ± 0.018	1.4 (I)
	2	0.204 ± 0.041	0.224 ± 0.014	1.1 (I)
GDNF (25 ng/ml)	1	0.966 ± 0.306	0.864 ± 0.075	0.9 (D)
	2	0.311 ± 0.094	0.589 ± 0.080	1.9 (I)
IL-6 (25 ng/ml)	1	0.942 ± 0.157	0.788 ± 0.269	0.8 (D)
	2	1.512 ± 0.117	2.426 ± 0.269	1.6 (I)
BDNF (25 ng/ml)	1	1.394 ± 0.220	0.846 ± 0.098	1.6 (D)
	2	1.526 ± 0.256	1.923 ± 0.589	1.3 (I)

Figure 4.14: Effect of growth factors on the CMV promoter. Adult DRG cultures were infected with AAV vectors containing the CMV promoter driving the luciferase reporter gene and maintained in the presence or absence of growth factors (GF). Cells were harvested and assayed for luciferase activity 7 days post-infection. Luciferase values are given as mean and std. error of quadruplicate wells for each sample. Fold increase or decrease represents the increase or decrease in expression induced by the addition of GF compared to cells that were maintained in the absence of GF (untreated).

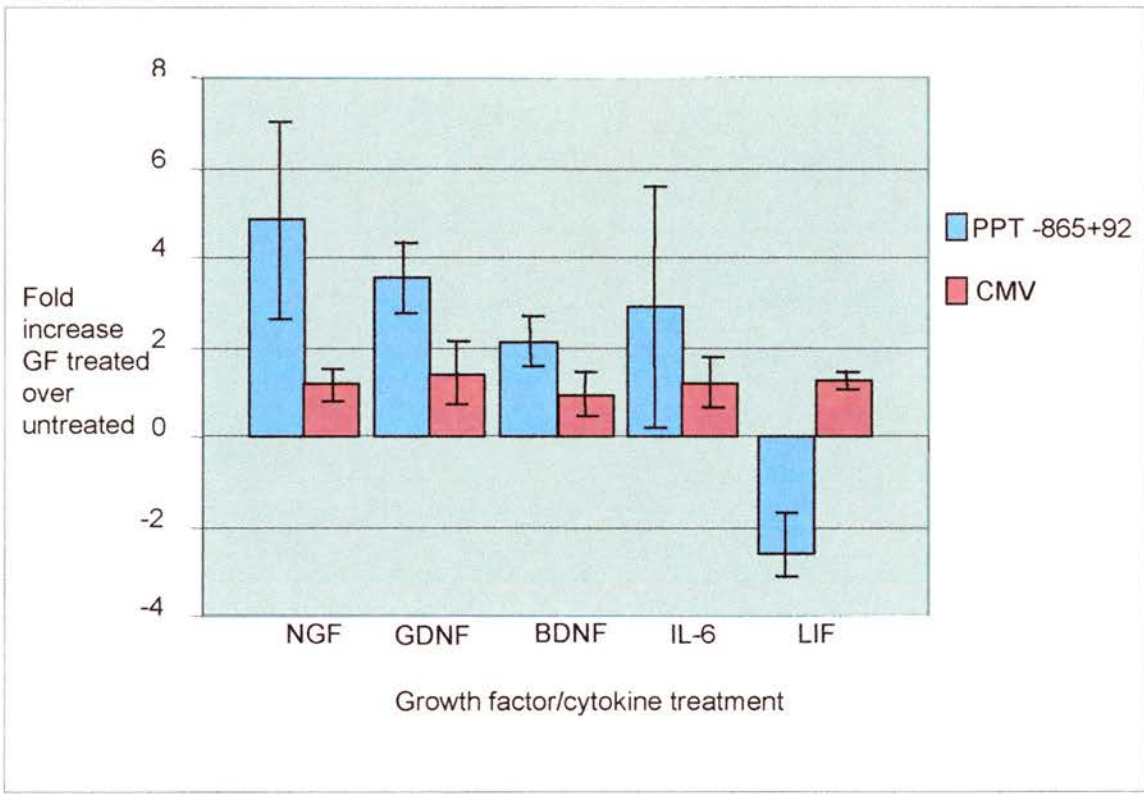

Figure 4.12: Effect of growth factors on the PPT-865+92 and CMV promoters. Adult DRG cultures were infected with AAV vectors containing the PPT promoter spanning base pair -865+92 or the CMV promoter driving the luciferase reporter gene and maintained in the presence or absence of growth factor (GF). Cells were harvested and assayed for luciferase activity 7 days post-infection. Values are given as fold increase or decrease in promoter activity induced by the addition of GF compared to samples maintained in the absence of GF. Data is expressed as mean ± SEM from two to five independent experiments, whereby each experiment incorporated triplicate or quadruplicate wells.

4.4.3 Discussion

It has been previously shown that exogenous NGF induces the PPT-A proximal promoter in cultured DRG neurons (Harrison *et al.*, 1999). This study set out to examine the effect of other growth factors on this promoter fragment spanning base pairs -865+92. Since adult DRG neurons survive in culture independently of added neurotrophic factors (Lindsay, 1988), the effect of trophic factors on promoter activity was investigated without the complications arising from variations in survival. To further ensure that the growth factors were acting specifically on the promoter the effect of these factors on the CMV promoter was also compared. This eliminated the possibility of growth factors exerting their effects by increasing survival or by influencing the AAV vector delivery system. The results of these studies are summarised in figure 4.12. It was found that NGF and GDNF induced luciferase actvity, reflecting an increase in PPT-A promoter activity whereas LIF acted to decrease PPT promoter levels in DRG neurons. The effects of BDNF, IL-6 and LIF and NGF together was not so obvious as in some of the experiments performed no effect was observed. To understand more about the signal transduction pathways that regulate the PPT-A gene and the regulatory elements involved, it is important to relate these promoter results to studies that have focused on examining the effect of growth factor on endogenous PPT-A gene expression *in vitro* and *in vivo*.

4.4.3.1 Action of NGF and LIF

Following nerve injury, neuropeptide expression in DRG neurons is seen to be altered: neuropeptides galanin and NPY are upregulated, whereas SP and CGRP

have been shown to be down-regulated (Zigmond *et al.*, 1996). Both LIF and NGF have been implicated as triggers for these changes in peptide phenotype since LIF expression is induced (Banner and Patterson, 1994; Curtis *et al.*, 1994) and target-derived factors such as NGF are lost following axotomy (Hokfelt *et al.*, 1994). In addition to having a role following nerve injury in the adult peripheral nervous system, NGF is thought to interact with nociceptive processing (Lewin and Mendell, 1993; Lewin and Barde, 1996). NGF can increase SP and PPT mRNA levels in DRG cells *in vitro* (Mulderry, 1994; Lindsay and Harmar, 1989; Jiang and Smith, 1995) and *in vivo* (Otten and Lorez, 1983; Vedder *et al.*, 1993). This suggests that NGF may be acting at the level of transcription to modulate these changes in expression. Harrison *et al.* (1999) examined the effect of NGF on the PPT-A proximal promoter spanning base pairs -865+92 and found a 30-fold increase in PPT-A promoter activity following treatment with NGF. In this thesis, the effect of NGF on PPT-A promoter activity was again examined and was found to induce promoter activity 2.5 to 7.8 fold. Although consistent with the work of Harrison *et al.* (1999) a large variation in the activation of promoter activity by NGF is apparent. This variability can be associated with a range of factors such as quality and quantity of DRG neuronal cultures, quality and titre of AAV prep used. This variability is apparent in all growth factor experiments performed, however it is particularly large when the effect of NGF alone and NGF together with LIF on promoter activity are examined.

One explanation for the variability is that nonneuronal cells within cultured primary neurons express LIF (Banner and Patterson, 1994; Sun and Zigmond, 1996a) and LIF is known to antagonise the effect of NGF (Verge *et al.*, 1995; Corness *et al.*, 1998; Mulderry, 1994). The percentage of satellite cells present in the cultures and

the amount of endogenous LIF expressed may therefore influence the levels of induction by NGF. Another consideration is that these primary cultures are thought to mimic axotomised populations of DRG neurons and following axotomy it is generally accepted that SP levels are decreased. However it has also been suggested that SP levels are increased in a population of neurons (Verge *et al.*, 1995). Therefore depending on the population of neurons present in the cultures, the overall result of induction observed may be affected by this.

As stated above, an essential consideration is the titre and also the method of preparation of AAV samples. Harrison *et al.* (1999) reported a 30-fold increase in promoter activity following treatment with NGF in DRG cultures. The work presented here also shows induced promoter activity, however this is on a much smaller scale (table 4.9). Harrison *et al.* generated virus vectors using the two plasmid-adenovirus helper method (section 1.4.7) and it is possible that this could have considerable influence on these studies. AAV vectors generated in this way often contain residual adenovirus and adenoviral proteins and generally result in lower titres of AAV (section 1.4.8). The presence of adenovirus is known to enhance expression from rAAV vectors (Fisher *et al.*, 1996). Furthermore, other contaminants in these AAV stocks can mimic transduction of AAV vectors and may be capable of generating artifactual results (Alexander *et al.*, 1997; Tenebaum *et al.*, 1999; personal observations, section 4.6.3).

LIF is also known to regulate peptide levels in sympathetic and sensory neurons. It has been shown that galanin induction in sensory neurons is LIF dependent, and LIF can regulate SP expression *in vivo* and in cultured sympathetic neurons (Nawa *et al*, 1991; Zigmond *et al.*, 1996; Zigmond and Sun, 1997).

Conflicting results regarding the effect of LIF on SP expression in sensory neurons have been reported. Zhang *et al.* (1995) report that high levels of exogenous LIF applied to cut sciatic nerve can significantly act to increase the percentage of SP mRNA positive cells, therefore preventing the reduction in SP associated with axotomy. However, experiments using LIF knock out mice suggest that LIF does not influence SP expression (Sun and Zigmond, 1996b) and *in vitro*, it has been shown that LIF alone has no effect on SP expression (Mulderry, 1994). In this thesis the effect of LIF alone on the PPT-A promoter was examined and found to decrease activity by 2 to 4 fold. Consistent with these results, Nawa *et al.* (1990) report that LIF acts to decrease SP expression in neuron enriched DRG cultures by 60%.

It has been reported that when exogenous LIF is applied together with NGF, the effect of NGF on SP expression is antagonised by the presence of LIF (Mulderry, 1994). When the effect of NGF and LIF together on the PPT-A promoter was investigated in this study, in two experiments a small increase in PPT-A promoter activity was observed compared to samples maintained in the absence of exogenous NGF and LIF. However, in another two experiments a decrease was seen and in another two experiments, no effect was observed in comparison to untreated. It is therefore not possible from these results to define what effect NGF and LIF together are having on the PPT-A promoter. However, this variation in promoter activity could again be explained by endogenous LIF expression in the cultures and LIF may still be performing this antagonistic role. The range of induction by NGF is variable, ranging from 2.5 to 7.8 fold and therefore the antagonist effect exerted by LIF in the presence of NGF will be highly variable. For example, if LIF and NGF are applied together and a 2 fold increase in promoter activity compared to untreated cells is

observed, this result may also be equivalent to a decrease in promoter activity when compared to an 8 fold induction of activity by NGF. In this set of experiments, the treatment with NGF alone was not included due to limitations of sample size. Such controls would therefore need to be performed before any conclusions could be drawn regarding the effects of LIF and NGF on the PPT-A promoter.

4.4.3.2 Action of GDNF

Much attention has been paid to the role of GDNF in sensory neurons as it exerts trophic effects on sensory neurons (Buj-Bello *et al.*, 1995; Matheson *et al.*, 1997; Moore *et al.*, 1996). It has been shown to be present in DRG neurons (Holstege *et al.*, 1998) and following axotomy, downregulation of GDNF in the dorsal horn is observed (Jongen *et al.*, 1999). This response is similar to that of SP expression following axotomy and GDNF immunoreactivity in the dorsal horn is similar to the expression pattern of SP (strong in laminae I and II-outer). This suggests that GDNF, like SP may have a role in nociception, and under normal nociceptive responses, GDNF may be acting to modulate SP.

When the effect of exogenous GDNF on the PPT-A promoter was studied, it was found that GDNF induced promoter actvity 2.4 to 4 fold. This is consistent with previous studies that examined the effect of exogenous GDNF on endogenous SP expression. Ogun-Muyiwa *et al.* (1999) showed a 3-fold increase in the percentage of SP mRNA expressing neurons and a 3-fold increase in the amount SP (measured by ELISA) in cultured DRG neurons following GDNF treatment. In addition, Adler (1998) found that the addition of exogenous GDNF to cultured DRGs increased endogenous SP concentrations by 3 fold. The effect of GDNF on the PPT-A

proximal promoter is consistent with effect of GDNF on the endogenous PPT-A gene. This is an important result and implies that the elements present on the proximal promoter spanning –865+92 are important for this induction of endogenous gene expression.

4.4.3.3 Action of IL-6

IL-6 may have a role in inflammation and the phenotypic responses associated with sensory neurons following axotomy. Peripheral nerve injury induces the production of IL-6 from Schwann cells (Bolin *et al.*, 1995) and DRG neurons (Murphy *et al.*, 1995). IL-6 mRNA and IL-6 receptor mRNA are highly expressed in rat (neonatal and adult) sensory and sympathetic ganglia (Gadient and Otten, 1996). Furthermore, 60% of IL-6 positive cells are colocalised with substance P and CGRP in DRG cell populations (Nordlind *et al.*, 2000) implying that DRGs may be responsive to IL-6. No studies so far have looked at the effect of IL-6 on endogenous PPT mRNA in DRG neurons, however like the other factors studied, IL-6 has trophic effects on DRG neurons and IL-6 is essential to modulate sensory functions *in vivo* (Gadient and Otten, 1997; Grud and Nelson, 1997; Campbell, 1998; Zhong *et al.*, 1999). In addition, IL-6 in high doses has been shown to induce SP mRNA in cultured sympathetic neurons (Fann and Patterson, 1994) and intrathecally applied IL-6 induces allodynia and hyperalgesia in rats (DeLeo *et al.*, 1996). These facts together suggest that IL-6 might act to regulate SP expression in DRG neurons. When the effect of exogenous IL-6 on the PPT-A promoter in DRG cultures was examined it was found that promoter activity was slightly induced. It would be useful to determine if IL-6 can regulate endogenous PPT-A mRNA before speculation can

be made regarding the role of IL-6 in regulating SP levels. However, IL-6 expression is induced following inflammation (Vallieres and Rivest, 1997) and inflammation also known to induce PPT-A mRNA in DRGs (Leslie *et al.*, 1995) therefore it is feasible to postulate that IL-6 may be involved in mediating this complex process. Studies to investigate the effect of IL-6 on endogenous PPT-A gene expression are ongoing.

4.4.3.4 Action of BDNF

Studies on the role of BDNF in the peripheral nervous system have focused on defining BDNF as a central modulator of pain (Thompson *et al.*, 1999). BDNF is constitutively expressed in DRG neurons (Ernfors *et al.*, 1990; Apfel *et al.*, 1996; Cho *et al.*, 1997) and it has been shown that this expression is associated with the NGF responsive population of neurons (Kashiba *et al.*, 1997b; Micheal *et al.*, 1997). Following inflammation BDNF expression is increased in DRG neurons in a NGF-dependent manner (Cho *et al.*, 1997) and this upregulated BDNF expression is thought to contribute to persistent inflammatory pain states (Mannion *et al.*, 1999; Kerr *et al.*, 1999). A change in BDNF expression is also seen following peripheral axotomy, whereby BDNF expression is increased in the large diameter population of DRG neurons and decreased in the small diameter nociceptive population (Tonra *et al.*, 1998). This is similar to the expression patterns of SP following both axotomy and inflammation. It was therefore thought that BDNF could have a role in influencing these changes in peptide expression in DRG neurons. The effect on BDNF on endogenous SP expression in cultured DRG neurons has been investigated and revealed that BDNF exerted no effect on expression or neuronal survival

(Mulderry, 1994). Mulderry suggested that this lack of effect on peptide expression could reflect the presence of significant quantities of endogenous BDNF in cultured DRG neurons since these DRGs themselves express BDNF.

The work carried out here examined the effect of BDNF on the PPT-A proximal promoter in cultured DRG neurons and a small increase in PPT-A promoter activity follwing BDNF treatment was observed in half the experiments performed. In the other experiments addition of BDNF did not influence PPT promoter activity. These contrasting results may be due to differences in cultures between experiments and reflect the presence of varying amounts of endogenous BDNF as Mulderry suggested. Under certain conditions, BDNF may influence regulation of the PPT-A promoter and under other conditions this regulatory effect is not found.

4.5 AAV vectors as a tool for studying transcriptional regulation of the PPT-A promoter in DRG cultures

The promoter experiments described have revealed possible functional elements involved in regulating the PPT-A promoter in both neonate and adult DRG cultures. In addition, it has shown that under certain conditions the PPT-A promoter is stimulus inducible and therefore contains important transcriptional elements that can mediate this effect. AAV vectors have been crucial for these studies, however various difficulties associated with this system of studying transient promoter activity delivered by virus vectors in heterogeneous primary neuronal cultures have also been highlighted. Two main difficulties have come to light. First the difficulties with generating and studying DRG cultures from adult rats and secondly, the lack of established controls when using the AAV vectors for transduction. Together these factors ultimately mean that high levels of variability were observed. Generating primary DRG cultures can be difficult and it is sometimes difficult to achieve high numbers of neuronal cells, making this a quite inconsistent technique. For example, over the course of this study a very large number of dissections were performed to produce adult DRG cultures (a minimum of 500) however, only 70% of these, at best would have provided successful cultures suitable for experimental use. In addition, because cells were maintained in culture for around 10 days, this time span meant that often cultures did not survive for numerous reasons such as contamination or unexplained death. Subsequently, from the number of infection experiments performed, only around 50% of these actually provided meaningful data. Therefore although use of DRG primary cultures is necessary for investigating promoter regulation in sensory neurons and can provide extremely interesting data, it is not an easy system to work with. Ideally, a cell line derived from DRG neurons may

provide insight into PPT-A promoter regulation in sensory neurons and eliminate these difficulties.

Another major factor associated with the use of DRG heterogeneous primary cultures for these experiments has been the difference between each set of cultures, including how the number of nonneuronal cells present in the cultures may be an important influencing factor when investigating stimulus inducibility. Although, nonneuronal cells are reduced by the method of culturing, they can never be completely eliminated and the fact that cells must be maintained in culture for 7 days adds to this difficulty. Schwann cells and fibroblasts are known to produce LIF and possibly other factors to affect phenotype. Therefore the presence of endogenous growth factors influencing outcome is always a possibility. In future experiments concerns of endogenous growth factors affecting results could be eliminated by first treating cultures with growth factor neutralising antibodies before addition of the exogenous growth factor. This way only the effect of the exogenous factor would be observed.

These cultures also introduce the complexity of studying unidentified intermediate molecules that are produced only under particular circumstances. For example, studies on SP expression in cultured sympathetic superior ganglia showed that IL-1 can increase SP expression only in cultures which contained nonneuronal cells implying the presence of an intermediate molecule (Freidin and Kessler, 1991). The problem of variability was not only restricted to the growth factor studies, as it was also apparent in the promoter analysis. The relative level of increase of promoter activity over the minimum promoter was varied and again this was likely to be a reflection of the cultures used for individual experiments.

Another major problem in this work was the lack of controls in order to normalise the luciferase values obtained to neuronal cell number and virus transduction efficiency, or to measure total protein concentration in order to express luciferase activity per mg of protein. This was due to the fact that the absolute protein concentration was too low to be accurately measured. In the future it would be useful to design vectors which express a marker molecule, perhaps via a functional IRES or to co-infect cells with another AAV vector expressing for example LacZ in order to normalise luciferase activity to β-gal activity. Although, this is not always possible due to the size constraints associated with AAV vectors.

Despite these highlighted problems, useful insight regarding regulation of the PPT-A promoter has been established and has provided a framework for future studies. This work has shown that this system of using AAV vectors to study promoter activity in cultured DRG neurons is a worthwhile system and could be greatly improved in the future. Attempts to establish an improved system have been carried out in parallel with these promoter studies (section 3.2). These studies have improved titres and purity of AAV preparations, and have investigated the infection of DRG neurons by AAV vectors, which contain the PPT promoter. In future, it would be beneficial to ensure all growth factor experiments are performed using the same known titre of AAV vectors, as this may also be a potential reason for variation between experiments. In addition, it may be advantageous to further purify the crude cell lysate to eliminate as many contaminating factors as possible.

By comparison between parameters such as promoter fragment or growth factor responses, it has not been possible to study quantitative values of promoter activity. However overall this system has been shown to be extremely useful for

investigating trends in promoter induction in response to various stimuli and determining potential transcriptional regulatory elements.

4.6 Miscellaneous and preliminary experiments

A number of experiments were performed which provided interesting preliminary data and if time permitted, these would have been repeated. These experiments were all concerned with the regulation of the PPT-A promoter in DRG neurons and in future may be interesting to follow up. The individual experiments will be described and the potential implications briefly discussed

4.6.1 Length of exposure to NGF may be important for promoter induction

For all previous growth factor work, DRG cultures were maintained in the appropriate growth factor for duration of the experiment. It is not known if this is necessary to achieve a response or whether only one exposure to the growth factor may be sufficient to cause an effect. This is relevant to the role of growth factors *in vivo* as chronic and acute exposure to NGF may define the physiological condition. Short term exposure to NGF may have a role in nociception whereby PPT-A gene expression is temporarily increased, whereas chronic exposure may be related to conditions such as arthritis in which PPT-A expression is upregulated over a long period of time.

4.6.1.1 Results

Adult DRG neurons were infected with AAV-865+92-LUC. Media containing NGF was added and removed at varying time points following infection. In all experiments cultures were harvested and assayed for luciferase activity 7 days post-infection as described in section 4.4. In experiment (1) NGF was added to the infected cultures 5 days post-infection (table 4.15). This experiment was only

performed once in duplicate. The results show that no NGF response was observed. In the following experiment, NGF was added immediately after infection, NGF was added 2 days post-infection or NGF was added 5 days post-infection. All were maintained in the additional NGF until cells were harvested on day 7 (table 4.16). Again this was performed once in duplicate. These preliminary results show that the PPT-A promoter is induced approximately 19 fold when NGF is added immediately after infection compared to untreated samples. This induction is reduced to around 3-fold when NGF is added 2 days following infection. In experiment (3), NGF was added immediately after infection. Cells were either maintained in NGF for the 7 day period or NGF was removed 3 days post-infection. Table 4.17 shows that when cells were treated with NGF for the 7 days there is a 14 fold increase in promoter activity compared to untreated cells and this induction is not seen in those samples where NGF was removed at day 3.

Luciferase activity	
NGF treated	Untreated
0.164	0.128
0.272	0.328

Table 4.15: Experiment 1. DRG cells were infected with AAV recombinant vectors containing the PPT-A promoter –865+82 driving the luciferase reporter gene. 5 days post-infection NGF was added to treated cells. Cultures were harvested and assayed for luciferase activity 7 days post-infection.

Luciferase activity			
NGF treatment day post-infection			Untreated
0	2	5	
67.56	11.85	4.337	3.5
67.77	9.64	4.397	3.74

Table 4.16: Experiment 2. DRG cells were infected with AAV recombinant vectors containing the PPT-A promoter –865+92 driving the luciferase reporter gene. NGF was added at varying times following infection.

Luciferase activity		
Maintained in NGF for 7 days	NGF removed at day 3	Untreated
16.83	0.272	0.121
14.76	0.102	0.105
3.08	-	0.07

Table 4.17: Experiment 3. DRG cells were infected with AAV recombinant vectors containing the PPT-A promoter –865+92 driving the luciferase reporter gene. NGF was added immediately after infection and then maintained in NGF for the duration of the experiment or removed at 3 days post-infection.

4.6.1.2 Discussion

It has been shown that NGF can induce the PPT-A proximal promoter in cultured adult DRG neurons (section 4.4.2.1). The preliminary results presented here suggest that to achieve this induction by NGF, cultures must be maintained in NGF for the 7 day infection period. Obviously, absolute conclusions cannot be drawn from the insufficient data presented here, particularly in light of the large variation observed between experiments. However, this is potentially an interesting area for future studies.

4.6.2 Effect of growth factors on the PPT-A promoter in neonate DRG cultures

It was also hoped to investigate the effect of growth factors on the PPT-A proximal promoter in neonate DRG cultures. However the difficulty associated with using neonate DRG cultures for such studies is that growth factors greatly influence the survival of the neurons therefore it is difficult to determine if the growth factor is truly having an effect. Numerous preliminary experiments were performed. The effects of GDNF, BDNF and LIF were investigated in addition to ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3). GDNF, BDNF and LIF have been described previously (section 4.4). NT-3 is a member of the NGF related family

of neurotrophins and like LIF, CNTF is a member of the cytokine family of growth factors (section 1.3). These growth factors were added in addition to NGF as neonate DRGs require NGF to survive in culture. Some experimental conditions were performed more than others therefore this growth factor study is incomplete and would have to be repeated before any conclusions could be drawn.

4.6.2.1 Results

Cultured DRG neurons were infected with AAV-865+92-LUC and maintained in medium containing NGF only or medium containing NGF in addition to other growth factors. The effect of LIF was examined three times in triplicate (table 4.16, experiments 1, 2 and 3). The data shown does not provide solid results regarding the role of LIF in neonate cultures. Experiment 1 and 2 suggest that LIF may act to increase promoter activity, however experiment 3 showed a reduction in luciferase activity in samples that were treated with NGF. Similarly, when the effect of GDNF was examined four times, each time in triplicate (experiments 2, 4, 5 and 7), conflicting results were obtained. Experiments 5 and 7 showed no difference between NGF only treated samples and samples treated with NGF together with GDNF. However experiments 2 and 4 showed an increase and decrease respectively in luciferase values compared to NGF only treated cultures. The effect of NT-3 together with NGF was examined twice in triplicate (experiments 6 and 8) and was shown not to influence luciferase values implying addition of NT-3 did not effect PPT-A promoter activity. When the effect of BDNF was examined (experiments 4, 5 and 6) more consistent results were observed. In the presence of BDNF in addition to

NGF, the luciferase values were reduced slightly compared to NGF only treated samples.

	NGF + Growth factor (GF)					
Fold increase (I) or Decrease(D) of NGF + GF treated over NGF only treated	Expt	LIF	BDNF	GDNF	GDNF+ BDNF	NT-3
	1	1.4 (I)	2.6 (D)	11.1 (I)	1.4 (I)	0
	2	4.8 (I)	1.8 (D)	3.6 (D)	2.3 (D)	0
	3	1.6 (D)	1.7 (D)	1.2 (I)	-	-

Table 4.18: Effect of growth factors on the PPT-A promoter in neonate DRG cultures. Neonate DRG cultures were infected with AAV vectors containing the PPT promoter (-865+92) driving the luciferase reporter gene and maintained NGF + presence or absence of growth factor (GF). Cells were harvested and assayed for luciferase activity 7 days post-infection. Data is represented as fold increase or decrease in expression induced by the addition of NGF+GF compared to 100 ng/ml NGF alone.

4.6.2.2 Discussion

This study set out to examine the effect of various growth factors on the PPT-A promoter in cultured neonate DRG neurons. The preliminary results revealed that this may be more difficult than initially planned compared to performing these experiments in adult cultures (section 4.4). The situation regarding neonate DRGs is much more complex since growth factors influence their survival. In addition, the molecular contents of neonate neurons change over the first and second postnatal weeks. This adds to the complexity when investigating the effects of growth factors since growth factor dependency and the presence of growth factor receptors change. For example, when the expression of NGF receptor and GDNF receptor was examined in newborn rat DRG neurons, it was found that in the first week of life, expression of the NGF receptor is downregulated. In contrast, expression of the

GDNF receptor is upregulated, changing the responsiveness of the neurons (Molliver and Snider, 1997; Molliver *et al.*, 1997; Bennett *et al.*, 1998b). As a result of these complexities, inconclusive and contrasting results were obtained. The only consistent result seen was when examining the effect of BDNF. The results showed that BDNF is acting to reduce luciferase activity and therefore possibly transcription from the PPT-A promoter. There are many ways BDNF could achieve this reduction in promoter activity. It may be that BDNF is antagonising NGF since they can act upon the same receptors or it may be due to activation of appropriate signal transduction pathways that can act specifically on the PPT-A promoter.

4.6.3 Potential problems associated with AAV vectors to investigate promoter regulation

In investigating regulation of the PPT-A promoter in adult and neonate DRG neurons (section 4.2), potential difficulties associated with using AAV vectors generated from the two plasmid-adenovirus method were encountered. Cultured neonate DRG neurons were infected with AAV preparation containing different PPT promoter fragments as described in section 4.2.2.2. On two occasions anomalous results were obtained and were thought to be associated with one particular preparation of AAV vectors.

4.6.3.1 Results

When the promoter activities of AAV-865+447-LUC, AAV-865+92, AAV-431+92 and AAV-47+92 were compared in both neonate DRG neurons as described in section 4.2.2, two surprising results were obtained. Cells were infected with the

AAV vectors, maintained in culture for 7 days, harvested and assayed for luciferase values as standard. However, when preparations of AAV from 25/4/00 were examined the promoter fragment –865+447, was found to be vastly increased. Induction over the minimum promoter was seen to be 675 and 9360 fold (table 4.19) in neonate DRG neurons. This same preparation of AAV was employed in three separate studies in adult DRG neurons. In contrast, a 31 to 116 fold induction of –865+92 promoter compared to the minimum promoter was observed, which was typical of all other studies in adult DRG neurons (see table 4.1, section 4.2.2.1).

Luciferase activity	Neonate	Expt	Promoter			Fold incr. -865 +447 over min.	Fold incr. -865+92 over min.
			-865+447	-865+92	-47+92		
		1	7666±1323	22.28±2.415	0.819±0.042	9360	27.2
		2	999±70.65	14.65±3.805	1.481±0.110	675	9.9
	Adult	1	32.46±18.92	3.807±0.342	0.28±0.047	116	13.6
		2	30.15±19.67	19.34±1.408	0.9±0.086	33.3	21.5
		3	26.02±5.702	10.64±1.405	0.84±0.298	31	12.7

Table 4.19: Expression of PPT-A promoter vectors in neonate and adult DRG neurons. DRG cultures were infected with AAV vectors from 25/4/00 containing the PPT-A promoter fragments –865+447, -865+92 and –47+92. Cells were maintained in medium for 7 days and then harvested and assayed for luciferase activity. The luciferase values shown are represented as mean and std. error of triplicate wells for each sample.

4.6.3.2 Discussion

It is unclear why these inconsistent results were observed compared to all other promoter data (section 4.2.2.). The only distinction between the experiments described above and all others in section 4.2.2, was the sample of AAV-865+447-LUC employed. This preparation was significantly different from all other samples as it had been generated by the two plasmid-adenovirus helper system whereas all

other AAV samples were generated from the three-plasmid system (section 1.4.8.1). It is unclear what caused this induction however it does seem to be unique to AAV vector expression in neonate DRG cultures as a similar increase in expression was not observed in adult DRG cultures infected with this sample (table 4.19). It was initially thought that it might be the result of contaminating adenovirus or viral proteins in the AAV stocks. To test this AAV-865+447-LUC, AAV-862+92 and AAV-47+92 were generated using both the three plasmid and the two plasmid-adenovirus methods for AAV production. The resulting AAV preparations were used to infect both adult and neonate DRG cultures and the activities compared. No induction in promoter activity was observed in those samples generated by the two plasmid-adenovirus system and the expression of promoter –865+447 was in accordance with all other experiments (data not shown).

Although this data is clearly aberrant, it has emphasised the future importance of employing highly purified AAV preparations to investigate transcriptional regulation of promoters. Many studies that employ AAV described in the literature actually generate rAAV preparations by this two plasmid-adenovirus method. The fact that AAV samples might contain factors, which under certain conditions have the potential to influence expression of the gene it is delivering, has important potential implications for future studies that use AAV. Therefore the possibility of artifactual results should be considered in the interpretation of studies involving AAV vectors, and these effects could be eliminated in future by improved purification procedures.

CHAPTER 5: GENERATION OF AAV VECTORS EXPRESSING PPT cDNA

5.1 Introduction

The purpose of this study was to generate AAV vectors that could be used as a tool to investigate the function of the PPT-A gene products *in vivo* and *in vitro*. The generation of mice in which the PPT-A gene was disrupted has allowed the function of the PPT-A gene to be defined *in vivo*. These studies have shown that when the PPT-A gene is disrupted in mice, although the fine detail is different between studies, generally the animals show reduced responses to painful stimuli and that local inflammatory responses were impaired (Zimmer *et al.*, 1998; Cao *et al.*, 1998; DeFelipe *et al.*, 1998). The use of virus vectors as an alternative or complementary method to transgenic analysis, in the manipulation of the nervous system has become increasingly popular. The availability of virus vectors that can express the PPT-A gene products would be a useful complement to the knockout mice available for investigating the function of this gene. The PPT-A knock out mice has defined the role of all the PPT-A gene products in mediating pain responses however most other studies investigating the function of the tackykinins have focused on the role of SP. Virus vectors may be a useful vehicle for studying specifically the role of SP and the other tachykinins individually. The creation of virus vectors, which express the desired gene products, can be delivered *in vivo* and can sustain long-term expression, would allow the individual gene products of the PPT-A gene to be investigated. In addition to complementation studies on mice in which the PPT-A gene has been disrupted, these vectors can be delivered to wild-type animals since overexpression of gene products can be as indicative of function as the generation of knockout mice (Magdaleno and Curran, 1999). The generation of virus vectors would also allow the

expression gene products to be targeted to specific tissues and allow analysis in other model systems in addition to mice.

These AAV vectors expressing the PPT-A gene products will also provide information about delivery of PPT-A gene products by AAV vectors and the protein requirements necessary for the regain of loss of neuronal function associated with the absence of SP. For example, SP expression has been studied in response to several neurodegenerative disorders and Parkison's (Gresch and Walker, 1999), Alzheimer's (Bouras *et al.*, 1990) and Huntington's (Richfield *et al.*, 1995) diseases are all associated with a progressive loss of PPT-A and SP expression in the brain. Information regarding the expression of exogenous SP in neurons would have important implications for future gene therapy protocols.

For these purposes AAV virus vectors were generated which contain both intact wild-type PPT cDNA and modified PPT cDNA, which lacks the sequences encoding SP, driven by either the PPT promoter (spanning base pairs –865 to +92) or the CMV promoter.

5.2 Generation of AAV vectors expressing PPT cDNA

5.2.1 Generation of rAAV vectors expressing PPT cDNA

In order to generate rAAV vectors that express the PPT-A gene products, NKA and SP, a cloning strategy was devised which would allow PPT cDNA expression to be driven by either the CMV or the PPT-A promoter. It has been shown that the CMV promoter can drive high levels of reporter gene expression in the context of AAV vectors (section 3.4.1) and therefore would allow high levels of expression of PPT cDNA in neurons and other cell types. It has also been shown that

the PPT-A promoter fragment spanning base pairs –865+92 contains many important *cis*-acting elements and can drive restricted reporter gene expression to populations of neurons and some nonneuronal cell types in cultured DRG cells (section 3.4.1).

Production of AAV constructs termed AAV-CMV-PPTcDNA and AAV-865+92-PPTcDNA that drive the PPT cDNA under control of the CMV and PPT promoters respectively required three cloning steps for each vector (figure 5.1 and section 2.2.4.1) due to the size restrictions imposed on AAV vectors. In order for constructs to be successfully packaged DNA fragments for insertion into pSub201 plasmids must be between 4.1 and 4.9 kb (section 1.4.8).

The first cloning step involved the replacement of the luciferase reporter genes of previously generated plasmids pGL3-CMV-LUC and pGL3-865+92-LUC with the PPT cDNA from plasmid pG1-βPPT cDNA (McGregor *et al.*, 1989) (cloning strategy described in detail section 2.2.4.1; figure 5.1). The DNA fragment consisting of promoter (CMV or –865+92) driving the PPT cDNA was then subcloned into plasmid pSnaB1-stuffer. This plasmid was constructed from the pGL3-basic backbone together with a stuffer DNA fragment derived from the mouse herpesvirus-68 (MHV-68) glycoprotein B gene which contains no known promoter or enhancer elements (Harrison *et al.*, 1999). This is a useful tool for the addition of so called 'stuffer' DNA to achieve the necessary size and incorporates the appropriate restriction sites for insertion into plasmid pSub201. The final cloning step inserted the DNA fragment consisting of the promoter driven PPTcDNA together with additional DNA into the pSub201 backbone to generate plasmids pSub201-CMV-PPTcDNA and pSub201-865+92-PPTcDNA.

Before converting these AAV packaging substrate plasmids into AAV particles it was first investigated whether these plasmids were functioning and expressing the appropriate products. Plasmid pSub201-CMV-PPTcDNA or no DNA was transfected into BHK cells (section 2.2.6.3). 2 days post-transfection mRNA was extracted (section 2.2.5.1) and RT-PCR analysis (section 2.2.5.2) was used to determine βPPT mRNA expression. In PCR reactions, plasmid pSub201-CMV-PPT was used as a DNA template as a positive control.

Since promoter fragment –865+92 does not support reporter gene expression in cell lines, plasmid pSub201-865+92-PPTcDNA could not be similarly analyzed. However the cloning steps were equivalent in the production of these constructs, and both restriction enzyme and sequencing analysis suggested no reason for this plasmid not to be viable. Figure 5.2 shows the results of this assay for mRNA expression from plasmid pSub201-CMV-PPTcDNA.

Plasmid pSub201-CMV-PPT was also analyzed for SP expression in cell culture. BHK cells were transfected with pSub201-CMV-PPT (section 2.2.6.2) and 48 h post-transfection cells were harvested and assayed for SP expression by SP immunoassay however no SP expression was found. SP immunoassay was also performed on DRG neurons to ensure the assay was functional (data not shown). It is conceivable that since endogenous SP is not expressed in this cell line and SP is produced as a result of both post-transcriptional and post-translational modification, expression of PPT cDNA may not processed correctly to generate the gene products.

The resulting plasmids pSub201-CMV-PPTcDNA and pSub201-865+92-PPTcDNA were used to produce rAAV (section 2.2.9.4).

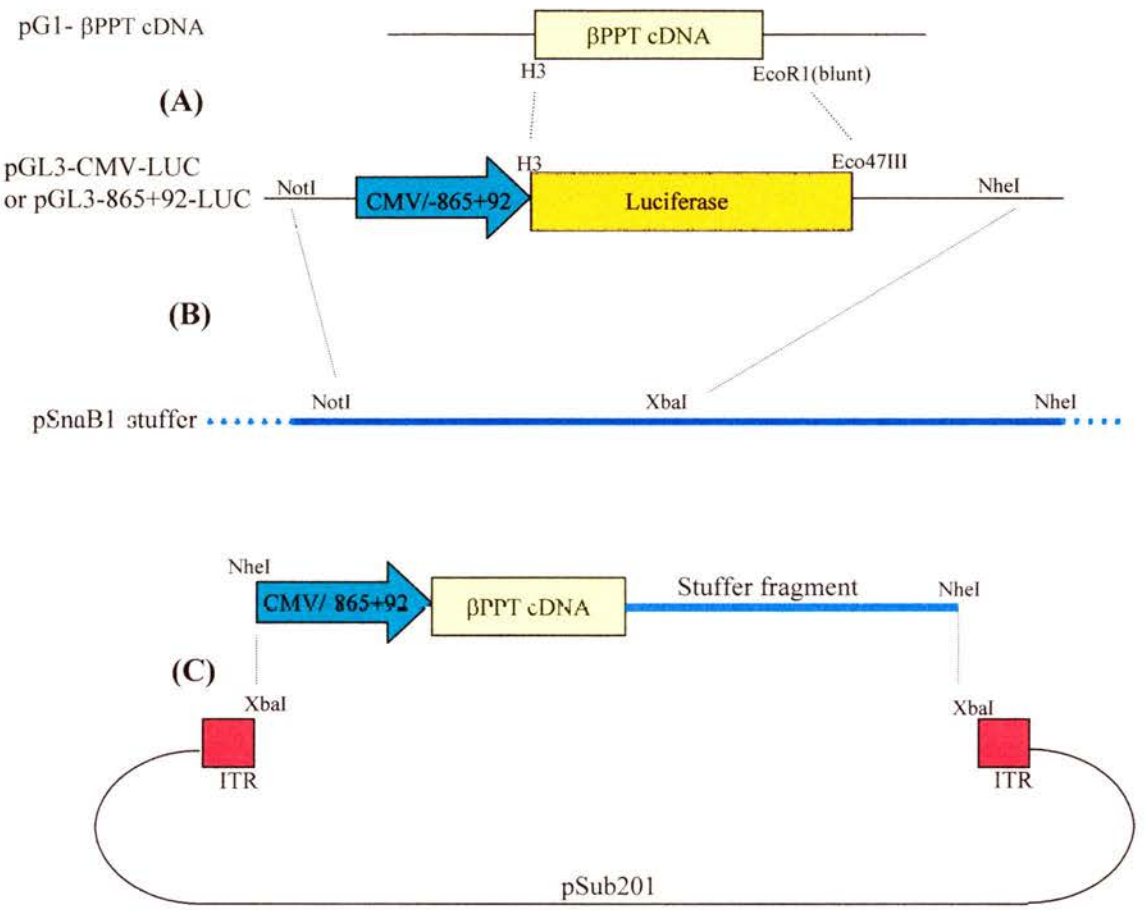

Figure 5.1: Three step cloning strategy to produce pSub201-CMV/PPT-βPPT for rAAV packaging. PPT cDNA was first inserted into either plasmid pGl3-CMV-LUC or pGl3-865+92-LUC. The cassette containing promoter, PPT cDNA and luciferase reporter gene was then subcloned into the stuffer plasmid. This DNA cassette containing additional DNA to make a 4.4 kb fragment was then transferred into plasmid pSub201.The resulting plasmids were termed pSub201-CMV-βPPT and pSub201-865+92-βPPT.

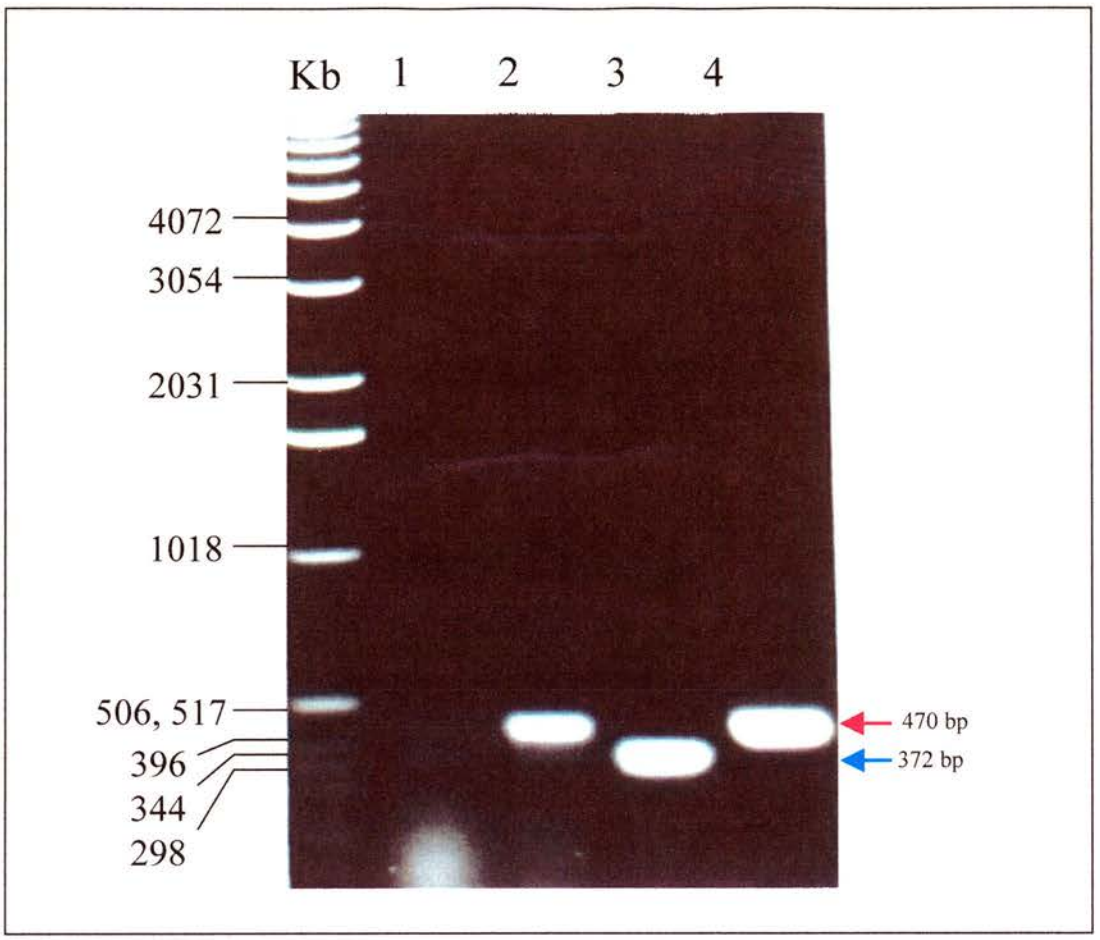

Figure 5.2 : Agarose gel electrophoresis of PCR products. BHK cells were transfected with pSub201-CMV-PPT and pSub201-CMV-PPTΔexon3 and RNA extracted 48h post-transfection. cDNA was synthesised from poly(A) RNA and amplified by PCR. Lane 1:-ve control (untransfected cells); 2: +ve control (plasmid pSub201-CMV-PPT; 3: pSub201-CMV-PPTΔexon3; 4: pSub201-CMV-PPT. Red arrow indicates the 470 bp PCR product from those cells transfected with pSub201-CMV-PPT, blue arrow represents 372 bp product from pSub201-CMV-PPTΔexon3 transfected cells.

5.2.2 Generation of rAAV plasmids expressing PPTcDNAΔexon3

To define more specifically the role of SP, a rAAV vector containing the PPT cDNA but lacking the specific SP encoding sequences was generated. Exon 3 contains the sequence information for SP and was therefore removed completely from the PPT cDNA. This was achieved by carrying out two separate PCR reactions that allowed the incorporation of a NsiI restriction enzyme site in both PCR products (sections 2.2.4.2 and 2.2.4.3; figure 5.3). The first PCR reaction amplified the region of DNA upstream of exon 3 and the second reaction amplified the DNA downstream. Subsequent digestion of the PCR products and simultaneous ligation of both PCR products into plasmid pCRII effected the removal of exon 3. This was confirmed by restriction enzyme digestion and sequence analysis of the mutated PPT cDNA sequence, which also verified that no secondary mutations in the cDNA had been introduced (figure 5.4).

The PPTΔexon3 fragment then replaced the wild-type PPT cDNA of plasmids pSub201-CMV-PPTcDNA and pSub201-865+92-PPTcDNA. The resulting plasmids were termed pSub201-CMV- PPTΔexon3 and pSub201-865+92-PPTΔexon3 (figure 5.3). Plasmid pSub201-CMV- PPTΔexon3 was analyzed as described above (section 5.2.1) for possible mRNA expression from PPTΔexon3. Figure 5.2 shows that when plasmid pSub201-CMV- PPTΔexon3 is transfected into BHK cells PPT mRNA is generated. The plasmids were then used for the production of AAV vectors.

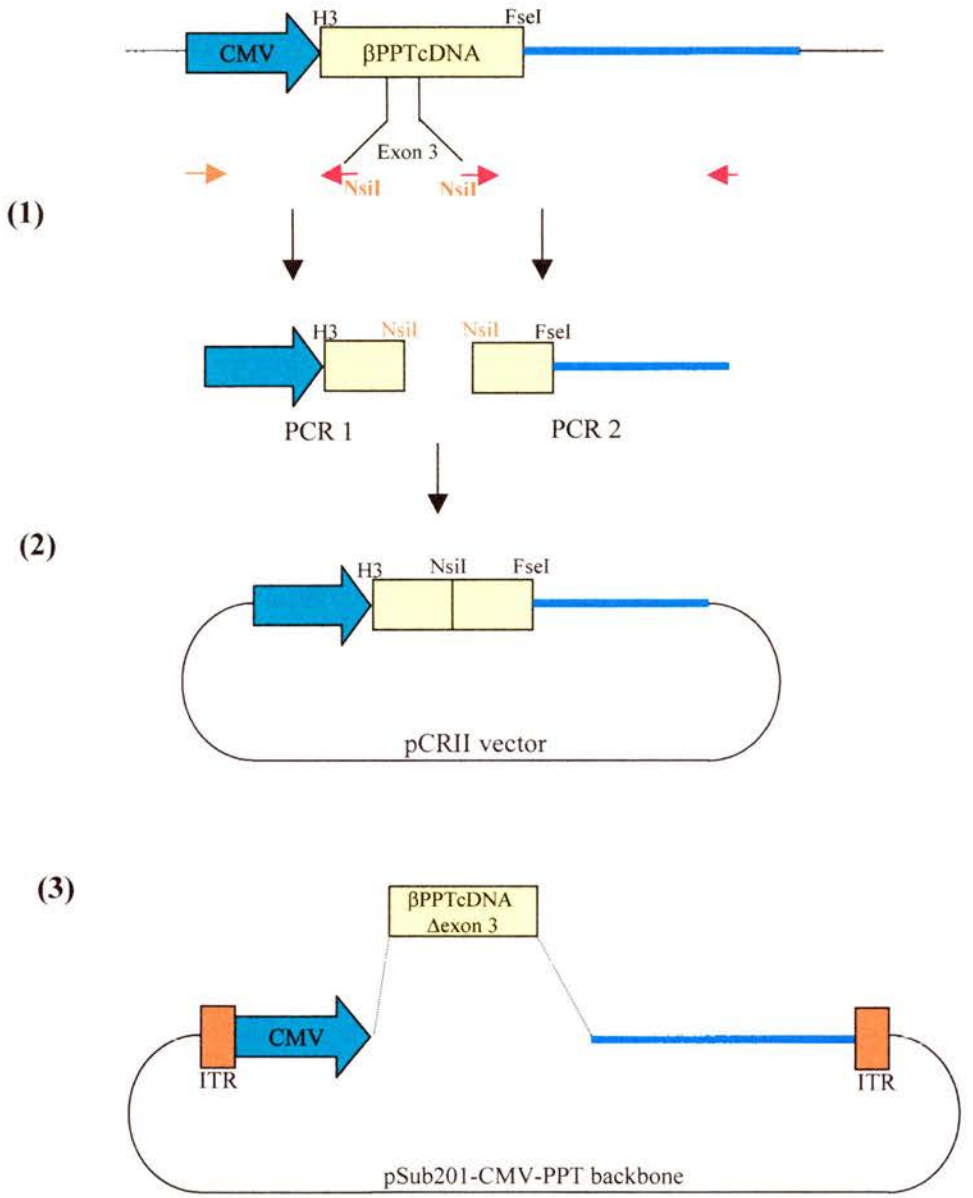

Figure 5.3: Strategy for the production of pSub201-CMV-PPT exon 3. (1) Two separate PCR reactions allowed the amplification of PPT cDNA upstream and downstream of exon 3 with the introduction of NsiI restriction enzyme sites. (2) Subsequent digestion of PCR fragments with NsiI and ligation into TA cloning vector pCRII resulted in the generation of PPT cDNA which lacked exon 3. (3) This mutant PPT fragment was then inserted into pSub201-CMV-PPT to replace the wild-type sequence.

(A)

CAAC ATG AAA ATC CTC GTG GCG GTG GCG GTC TTT TTT CTC GTT TCC ACT CAA
CTG TTT GCA GAG GAA ATC GGT GCC AAC GAT GAT CTA AAT TAT TGG TCC GAC
TGG TTC GAC AGT GAC CAA ATC AAG GAG GCA ATG CCG GAG CCC TTT GAG CAT CTT
CTT CAG AGA ATC GCG CGA AGA CCC AAG CCT CAG CAG TTC TTT GGA TTA ATG GGC
AAA CGG GAT GCT GAT TCC TCA ATT GAA AAA CAA GTG GCC CTG TTA AAG GCT CCT
TAT GGG CAT GTG CAG ATC TCT CAC AAA AGG CAT AAA ACA GAT TCC TTT GTT GGA
CTA ATG GGC AAA AGA GCT TTA AAT TCT GTG GCT TAT GAA AGA AGC GCA ATG CAG
AAC TAC GAA AGA AGG CGT AAA
TAAACCCTGTAACGCACTACTTATTCATCTCCATCTGGTCCGCGAG

(B)

CAAC ATG AAA ATC CTC GTG GCG GTG GCG GTC TTT TTT CTC GTT TCC ACT CAA
CTG TTT GCA GAG GAA ATC GGT GCC AAC GAT GAT CTA AAT TAT TGG TCC GAC TGG
TTC GAC AGT GAC CAA ATC AAG GAG GCA **ATG CAT** AAA CGG GAT GCT GAT TCC TCA
ATT GAA AAA CAA GTG GCC CTG TTA AAG GCT CTT TAT GGG CAT GGT CAG ATC TCT
CAC AAA AGG CAT AAA ACA GAT TCC TTT GTT GGA CTA ATG GGC AAA AGA GCT TTA
AAT TCT GTG GCT TAT GAA AGA aGC GCA ATG CAG TAC TAC GAA AGA AGG CGT AAA
TaaaCCCTGTAACGCACTATCTATTCATCTCCATCTGTGTCCGGAT

Figure 5.4: Nucleotide sequence of cDNA coding for (A) wild-type PPT and (B) mutant PPTΔexon3. Underlined nucleotides represent exon 3. In wild-type sequence red nucleotides indicate bases that were mutagenised to produce NsiI restriction enzyme site (ATGCAT). Bold bases in mutant sequence show resulting NsiI site following removal of exon 3.

5.2.3 Expression of SP from AAV-CMV-PPTcDNA

To investigate whether cells infected with AAV-CMV-PPTcDNA actually express SP, adult DRG neurons were cultured (section 2.2.7.1) and infected with increasing amounts of virus (section 2.2.10.1). No virus, $1x10^6$, $5x10^6$ and $1x10^7$ infectious particles of AAV-CMV-PPTcDNA were each used to infect approximately 500 neurons. Infected cells were maintained in culture for 7 days and then immunostained for SP expression (section 2.2.5.3). DRG cultures were also infected with the same amounts of AAV-CMV-PPTΔexon3 as a control experiment. Figure 5.5(a) shows that when no virus was used, antibodies against SP showed staining in only a small population of neurons, which is compatible with the expected expression pattern for SP whereby approximately 20 to 30% of DRG neurons are SP positive. When increasing concentrations of AAV-CMV-PPTcDNA were used for infection, SP expression increased, particularly in the nonneuronal population (figure 5.5 (b)-(d)). Cells infected with AAV-CMV-PPTΔexon3 showed similar expression levels to those cells in which no virus was added (figure 5.5 (a) and (e)). This experiment was not performed using AAV-865+92-PPTcDNA and AAV-865+92-PPTΔexon3. Since expression from the PPT promoter is greatly restricted in DRG neurons (section 3.4.2), it is highly probable that expression of endogenous SP from the DRG cultures could not be distinguished from SP expression as a result of virus infection. Ideally this experiment should have been performed in DRG cultures from mice that lack the PPT-A gene to ensure SP staining is a result of virally infected cells.

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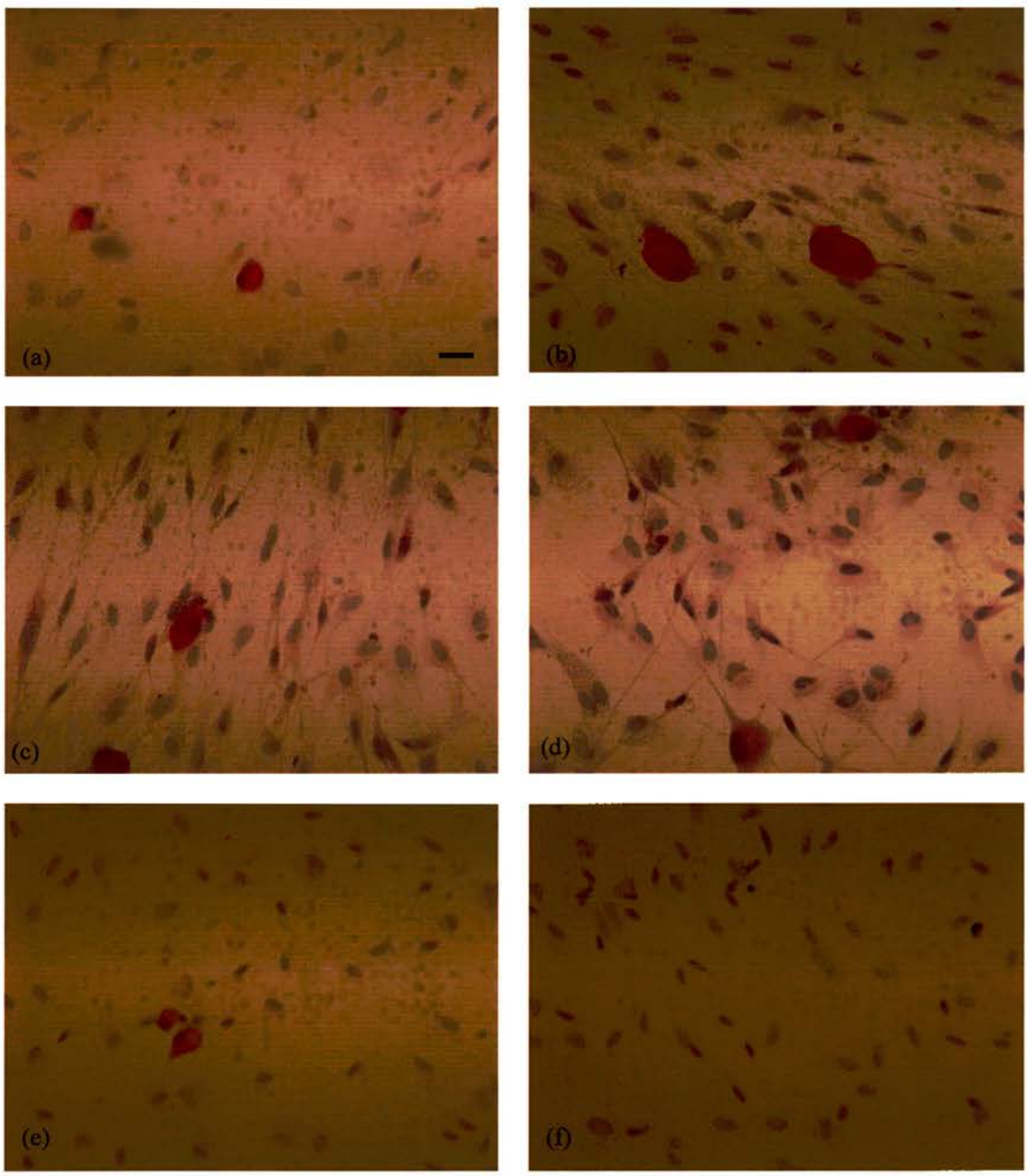

Figure 5.5: Adult DRGs infected with (a) no virus, (b) $1x10^6$, (c) $5x10^6$ and (d) $1x10^7$ infectious particles of AAV-CMV-PPTcDNA and immunostained for SP. Those cells positive for SP expression are pink in colour. DRGs were also infected with increasing concentrations of AAV-CMV-PPTΔexon3. (e) shows cells infected with $1x10^7$ infectious particles of AAV-CMV-PPTΔexon3 and (f) $1x10^7$ infectious particles of AAV-CMV-PPTcDNA when primary antibody was omitted as a negative control. All pictures shown are 25 x magnification and scale = 25 μm

5.2.4 Discussion

pSub201 plasmids were generated that contain the PPT promoter or the CMV promoter driving intact wild-type PPTcDNA or mutant PPTcDNA. These were used to produce AAV vectors and termed AAV-CMV-PPTcDNA, AAV-CMV-PPTΔexon3, AAV-865+92-PPTcDNA and AAV-865+92-PPTcDNA Δexon3. One AAV vector, AAV-CMV-PPTcDNA was shown to express SP upon infection of cultured DRG neurons and this suggested that all other AAV vectors would be capable of expressing SP. As DRG neurons express endogenous SP, it is difficult to prove that the SP expression detected is a result of expression from the AAV vectors. However increasing concentrations of AAV-CMV-PPT did correlate with increased SP immunostaining in nonneuronal cells. This shows that SP is capable of being expressed in noneuronal cells of DRG, although it was also found that SP was not expressed in BHK cells. Therefore, unlike BHK cells, noneuronal cells populations are capable of processing the PPT-A pro-peptide and expressing SP. This has important implications for understanding the expression and role of SP in nonneuronal cells *in vivo*.

Unequivocal evidence of SP expression from the rAAV vectors would have been provided by infection of cultured DRG neurons from the PPT-A knockout mice. Many attempts were made to culture DRG neurons from these mice, however difficulty was encountered in achieving healthy cells for used in these experiments. As a result of this, expression from AAV-865+92-PPTcDNA could not be examined. If future attempts to generate the DRG cultures from the knockout mice prove problematic, other options could be considered to examine expression from AAV-865+92-PPTcDNA. For example, intact ganglia or other neuronal populations of

embryonic origin from PPT-A knockout mice could be cultured, if these prove more successful. Future experiments should also include determination if AAV-CMV-PPTΔexon3 expresses NKA, however at the time this work was performed, no good commercial antibodies for this peptide were available.

The generation and partial validation of these AAV vectors means that valuable tools will be available for investigating the role of the PPT-A gene products. It is hoped these vectors will be used in a variety of experiments not only to define the tachykinins function in nociceptive pathways but in other physiological functions such as depression and anxiety. The availability of PPT-A gene knockout mice, whose phenotype has been described (Ciao *et al.*, 1998; Zimmer *et al.*, 1998) will allow the AAV-CMV-PPT vectors be used to attempt to rescue phenotypic characteristics associated with these mice. As an alternative approach, overexpression of the PPT-A gene *in vivo* in wild-type animals might provide a clearer understanding of the function of the various PPT-A gene products (Magdaleno and Curran, 1999).

The generation of PPT cDNA expressing vectors, which are driven by the PPT promoter, will allow expression to be restricted to various populations in DRG cultures and possibly *in vivo*. This might be useful for determining the role of SP in neurons compared to nonneuronal cells in mixed primary cultures or heterogenous tissue *in vivo*. Under basal conditions SP expression is limited to neurons however it has been shown in this thesis that nonneuronal cells can express SP and it known that SP is expressed in glial cells under certain conditions such as nerve injury (Too *et al.*, 1994; Lin *et al.*, 1995). This might indicate a differential function for SP in neurons

and noneuronal cells and the production of these AAV vectors could contribute to understanding the role of SP in noneuronal cells.

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

The work presented in this thesis describes the use of AAV vectors as a tool for the transduction of recombinant DNA constructs into cultured DRG neurons. This subsequently allowed transcriptional regulation of the PPT-A promoter to be investigated. The initial aims of this study were to optimise the conditions of AAV production and investigate the transduction of DRG neurons by these vectors. AAV vectors containing either the CMV promoter or PPT promoter (-865 to +92) were able to drive long-term expression of the GFP reporter gene in DRG cultures. All cell types in a heterogenous primary culture of DRG neurons infected with AAV-CMV-GFP showed high expression levels, which increased linearly with virus dose. Those cells infected with AAV-PPT-GFP showed a more restricted expression pattern whereby expression was mainly associated with neuronal populations. In addition, as higher doses of AAV-PPT-GFP virus particles were used for infection of DRG cultures, the number of GFP expressing cells was not similarly increased. This highlighted the more restricted expression pattern of the PPT-A promoter that has been previously reported (section 1.2.3) and provided important information about the expression pattern of CMV and PPT promoter fragments in cultured DRG when delivered as a rAAV particle. These studies may also have important implications for the generation of future vectors for gene therapy. AAV is a highly 'promiscuous' virus and can infect a wide variety of cell types. Although this is considered an advantage of AAV vectors for use in gene therapy, it may also be negative if a restricted expression pattern is desired. Studies have focused on targeting expression from AAV vectors to particular cell types (section 1.4.8.4) and one way of achieving

this is by the use of promoter fragments that have a limited expression pattern. The CMV promoter, although directing high expression levels, will drive expression in all cell types therefore would not be a suitable candidate for cell specific expression in the context of AAV vectors. As described above, the PPT-A promoter was observed to have a restricted expression pattern in cultured DRG neurons. The studies described have provided the basis for future analysis of cell types that can support activity of the PPT-A promoter and determine if it has the necessary requirements for driving neuronal specific expression.

Further knowledge of the expression pattern of the PPT-A promoter would be increased by a greater understanding of the influences, which contribute to the regulation of the PPT-A promoter. This was addressed by the second aim of this thesis, which was to investigate the transcriptional regulation of the PPT-A promoter in DRG neurons. AAV vectors containing deletion fragments of the PPT-A promoter were used for infection of both adult and neonate DRG neurons. It was established that in the presence of NGF, different promoter fragments were differentially regulated between adult and neonate DRG neurons, perhaps reflecting the normal plasticity of PPT-A expression observed *in vivo*. Potential enhancer and silencer sequences were identified and provided important information about the promoter elements that contribute to PPT-A promoter activity and gene expression in a tissue specific and stimulus inducible manner. The region between +92 and +447 was shown to possess enhancer activity in adult DRG neurons but was not observed to be similarly active in neonate DRGs. In addition, in adult DRG neurons, the region between base pairs –484 to –47 possessed further enhancer elements. In contrast, in neonate DRG neurons potential repressor activity in this domain was observed.

To investigate the stimulus inducible regulation of the PPT-A promoter, the effect of various growth factors on the PPT-A promoter fragment –865 to +92 was studied in adult DRG neurons. The effects of NGF, BDNF, GDNF, IL-6 and LIF on PPT-A promoter activity were analysed. These growth factors were selected on the basis that they are thought to modulate SP or PPT-A expression under basal conditions or in response to nerve injury (section 1.3.1). It was found that both NGF and GDNF acted to induce PPT-A promoter activity, indicating the presence of regulatory elements on this promoter fragment –865+92 that can mediate this effect. This is similar to the effects of NGF and GDNF on endogenous PPT-A gene expression. NGF is known to regulate PPT-A mRNA and SP expression (Lindsay and Harmar, 1989; Mulderry, 1994; Leslie *et al.*, 1995) and GDNF can induce SP levels in DRG cultures (Ogun-Muyiwa *et al.*, 1999; Adler, 1998). In addition, LIF was observed to decrease PPT-A promoter activity in cultured DRG neurons. Again this suggested that elements of the PPT-A promoter are responsive to the presence of this growth factor. It is unclear what effect LIF has on endogenous PPT-A expression in DRG neurons, however one study has demonstrated that LIF can decrease SP expression (Nawa *et al.*, 1990).

These studies provided important information about the regulation of the PPT-A promoter and the use of AAV vectors was central to performing this work. The only other method for successfully transfecting DRG neurons is microinjection of plasmid DNA into cells however this system does not allow the investigation of promoter inducibility by growth factors (Fiskerstrand and Quinn, 1995; Quinn *et al.*, 2000). In contrast, when PPT-A promoter fragments are delivered as a recombinant AAV vector the PPT-A promoter can be activated or repressed by stimulus. However

the problems associated with this experimental system were highlighted. It was initially believed that crude cell lysate would be suitable for the transduction of AAV vectors into cultured DRG neurons. It became apparent over the course of this study that it may be beneficial to the quality of future data to ensure that AAV preparations are further purified. With the improvements and simplifications in protocols for the purification of AAV vectors this can be easily achieved for future work.

A final aim of this thesis was to create AAV vectors, which could be used to investigate the function of the PPT-A gene products *in vitro* and *in vivo*. AAV vectors containing the PPT-A promoter (-865+92) or the CMV promoter driving intact PPT cDNA or mutated cDNA, which lacked the sequence information for producing SP, were generated. These AAV vectors were shown to be functional in that peptide was produced but further analysis would be required. Mice which, lack the PPT-A gene, have been previously generated (Ciao *et al.*, 1998; Zimmer *et al.*, 1998) and it is hoped that these virus vectors could be an important complement to these animals to define a role for either SP or NKA. These vectors would also be useful for overexpression of tachykinins in wild-type animals.

6.2 Future directions

The results obtained from this study, using AAV vectors to investigate regulation of the PPT-A promoter, will lead to a number of further studies aimed at both improving the experimental system and our understanding of regulation of the PPT-A gene at the transcriptional level.

The investigation of the promoter deletion constructs provided valuable information into potential regulatory regions of the promoter, that are involved in the

regulation of the PPT-A promoter in cultured DRG neurons. The generation of site-specific mutants in future studies would allow the specific elements of the PPT-A promoter that are involved in tissue specific expression to be defined. In addition, the deletion constructs described in this study could be used to define the regions that are responsible for mediating the effects of GDNF, NGF and LIF. This could be achieved by investigating the promoter activities of each of these PPT-A promoter fragments in the presence or absence of growth factors. It may also be interesting to investigate the effects of different concentrations of exogenous growth factor and the timing of addition to the AAV infected DRG cultures. One set of preliminary experiments suggested that the long-term exposure (for the 7 day infection period) might be necessary to achieve maximum PPT-A promoter induction in cultured DRG neurons by NGF. Such studies could have important implications with respect to induction of PPT-A gene expression *in vivo*. Long-term exposure to NGF and therefore long-term upregulation of PPT-A gene expression may occur through elements that are distinct from those involved in short-term exposure.

The generation of PPT cDNA expressing AAV vectors gives rise to many potential studies. These AAV vectors could be used to investigate the function of all PPT gene products and specifically SP in sensory neurons and other cell types that express endogenous PPT-A. For example, characteristic phenotypes have been described for mice that lack the PPT-A gene. The PPT-A gene products could be delivered *in vivo* by these AAV to determine if the phenotypes are complemented by expression of the tachykinins from the AAV vectors. Additionally, delivery of the AAV vectors, which express the mutated PPT cDNA, to these transgenic mice will determine the specific role of SP in generating these phenotypes. Another approach

would be the overexpression of the tachykinins by these AAV vectors in wild-type animals and examination of the resulting phenotype to give more insight into their functions. At a cellular level, overexpression in cultured DRG neurons and other cell types is likely to generate changes in electrophysiology or other marker molecules, again contributing to defining the role of the PPT-A gene products. PPT cDNA expressing AAV vectors driven by the PPT promoter –865+92, could be used to restrict expression to neuronal populations. Together it is hoped that these vectors will provide important information about using AAV to deliver a protein that has been lost in disease, for example loss of SP expression has been associated with degenerative diseases. Therefore this has particular importance to the field of gene therapy and therapeutic protein delivery.

In this thesis it was also established that the PPT promoter spanning nucleotides –865 to +92 demonstrated a more restricted expression pattern than that of the CMV promoter. To further investigate the populations of DRG neurons that specifically support PPT promoter activity it would be useful to exploit the AAV vectors which contain the PPT promoter driving the GFP reporter gene. GFP expression could be analysed in conjunction with immunostaining of marker molecules to define cell types and neuronal populations that can support promoter activity.

It became apparent over the course of this study, that the use of highly purified AAV preparations will be essential for future studies both *in vitro* and *in vivo.* The presence of contaminants in AAV stocks might influence the PPT-A promoter data generated and contaminants are known to give artefactual results (Alexander *et al.*, 1997). Essential future work should involve the establishment of a

protocol for a purification procedure that can be performed routinely, and many such methods have been described over the course of this thesis (section 1.4.8.5). It is believed that this would make a marked improvement to the quality and quantity of data generated, using AAV to investigate regulation of the PPT-A promoter in DRG neurons.

Appendix 1: Comparison of promoter deletion constructs – Raw luciferase values

The promoter activity of AAV vectors containing each promoter was examined three times in each experiment in both adult and neonate DRG cultures. Each experiment differs in the promoter fragments that are compared. Tables show the raw luciferase and mean values form each experiment, together with the AAV preparation and the amount of virus that was used in each experiment.

Expt	AAV prep	Amt (infectious particles or µl)	Promoter	Luciferase values (relative light units)				
				1	2	3	4	Mean
1	24/11/00	20µl	-865+92	2.272	1.990	2.899	-	2.387
			-671+92	7.708	3.556	3.515	-	4.926
			-431+92	2.846	5.526	5.179	-	4.516
2	24/11/00	20µl	-431+92	1.045	0.225	0.349	-	0.54
			-345+92	0.799	0.487	0.684	-	0.657
			-47+92	N/d	N/d	0.038	-	0.013
3	24/11/00	$5x10^5$ particles	-865+447	2.123	0.999	0.084	-	1.069
			-865+92	1.259	0.208	0.128	-	0.532
			-671+92	0.065	N/d	N/d	-	0.022
4	4/8/00	8µl	-865+92	45.39	29.63	50.01	-	41.68
			-431+92*	81.64	69.74	112.4	-	87.93
			-47+92	1.471	1.706	-	-	1.59
5	4/8/00	8µl	-865+92	13.34	14.52	28.12	-	18.66
			431+92**	23.07	40.76	51.79	-	38.54
			-47+92	0.477	0.376	0.38	-	0.411
6	25/4/00*	12µl	-865+447	26.25	3.18	3.032	-	10.82
			-865+92	5.036	5.016	3.538	-	4.53
			-47+92	0.296	0.178	0.373	-	0.28
7	25/4/00*	12µl	-865+447	25.91	4.211	0.037	-	10.05
			-865+92	60.62	7.155	1.258	-	23.01
			-47+92	1.032	0.996	0.677	-	0.9
8	25/4/00*	12µl	-865+447	6.17	6.532	13.32	-	8.674
			-865+92	11.3	9.999	16.68	-	12.66
			-47+92	1.437	0.902	0.182	-	0.84
9	25/4/00*	15 µl	-865+92	3.891	6.376	0.537	-	3.60
			-671+92	3.279	0.809	3.604	-	2.56
			-47+92	0.651	0.124	0.308	-	0.361
10	4/8/00	15µl	-865+447	22.95	14.22	21.67	-	19.61
			-865+92	30.55	50.84	26.16	-	35.83
			-47+92	1.203	1.656	1.293	-	1.384
11	4/8/00	15µl	-865+447	70.62	25.95	45.99	-	47.54
			-865+92	62.94	101.7	84.97	-	83.2
			-47+92	1.774	0.945	1.351	-	1.36
12	25/4/00	15µl	-865+92	0.34	0.267	0.036	-	0.214
			-671+92	0.41	0.24	0.094	-	0.248
			-47+92	0.08	0.028	0.129	-	0.079

13	24/11/00	$7x10^6$	-671+92	0.728	1.771	0.078	-	0.859
		particles	-431+92	0.33	0.459	0.936	-	0.575
	26/2/01		-47+92	0.42	0.131		-	0.276
14	24/11/00	$7x10^6$	-671+92	1.727	1.358	0.478	-	1.188
		particles	-431+92	2.564	0.948	-	-	1.756
	26/2/01		-47+92	0.204	0.153	0.04	-	0.132
15	24/11/00	$7x10^6$	-671+92	0.334	0.32	-	-	0.327
		particles	-431+92	0.895	0.275	-	-	0.585
	26/2/01		-47+92	0.107	0.056	0.032	-	0.065
16	24/11/00	15µl	-865+447	0.188	0.283	0.497	0.167	0.284
			-671+92	0.032	0.091	0.037	0.007	0.042
17	22/9/00	15µl	-865+447	0.097	0.055	0.261	0.139	0.138
	24/11/00		-431+92	0.072	0.008	0.045	0.073	0.050
18	22/9/00	15µl	-865+447	0.029	0.005	0.030	0.059	0.031
	24/11/00		-431+92	0.062	0.137	0.043	0.089	0.083

Table A1.1: Actual raw luciferase mean and values obtained for each experiment performed in **adult** DRG cultures.

Expt	AAV prep	Amt (µl)	Promoter	Luciferase values (relative light units)				
				1	2	3	4	Mean
1	4/8/00	12	-865+447	106.4	126.6	132	-	121.7
			-865+92	208.9	393.9	226	-	276.1
			-431+92	64.66	83.83	58.31	48.01	63.70
			-47+91	16.9	16.32	-	-	16.16
2	4/8/00	12	-865+447	79.41	73	61.07	-	71.16
			-865+92	136.3	117.9	75.63	-	109.9
			-431+92	40.82	39.85	-	-	40.34
			-47+92	11.99	15.44	11.29	-	11.57
3	24/11/00	20	-865+447	11.31	22.66	11.76	-	19.70
			-865+92	59.3	68.5	-	-	63.9
			-671+92	75.22	26.2	-	-	50.71
			-431+92	55.63	93.3	6.5	-	51.81
			-345+92	51.15	72.44	28.10	-	50.56
4	24/11/00	20	-865+447	89.29	23.22	50.26	-	54.26
			-865+92	149.7	142	159	-	150.23
			-671+92	112.1	97.26	65.88	-	91.75
			-431+92	10.79	18.81	214.9	-	81.5
			-345+92	274.9	198.6	20.41	-	164.64
			-47+92	3.606	51.1	28.84	-	27.85
5	25/4/00*	20	-865+447	1678	2440	2260	4010	4014
			-865+92	27.92	20.23	25.44	15.54	22.28
			-431+92	3.33	3.774	2.788	2.448	3.085
			-47+92	0.884	0.691	0.906	0.796	0.819
6	25/4/00*	20	-865+447	405	330	273	324	333
			-865+92	12.5	27.52	10.71	7.86	14.65
			-431+92	3.125	2.078	4.690	3.339	3.308
			-47+92	1.156	1.101	1.549	2.205	1.481

Table A1.2: Actual raw luciferase and mean values obtained for each experiment performed in **neonate** DRG cultures.

Expt	AAV prep	Amt (infect-ious particles or μl)	Promoter	Luciferase values			
				1	2	3	Mean
1	24/11/00	20ul	-865+92	0.98	0.648	1.228	0.952
			-60mut	0.051	0.17	0.112	0.111
			-47+92	0.124	0.178	0.158	0.152
2	24/11/00	20ul	-865+92	0.082	0.723	-	0.403
			-60mut	0.033	0.071	-	0.052
			-47+92	0.038	0.043	0.024	0.035
3	24/11/00	20ul	-865+92	10.22	16.47	4.93	10.54
			-60mut	3.26	3.07	2.65	2.99
			-47+92	2.934	1.04	1.57	1.85
4	25/2/01	2.5×10^6	-865+92	2.012	5.770	0.915	2.899
			-60mut	1.919	3.716	1.546	2.394
			-47+92	0.620	0.842	0.626	0.631

Table A1.3: Actual raw luciferase values – comparison of promoter fragments –865+92, -60mut, -47+92 in the presence NGF Adult DRG cultures.

Expt	AAV prep	Amt (infect-ious particles or μl)	Promoter	Luciferase values			
				1	2	3	Mean
1	24/11/00	20 ul	-865+92	1.178	1.558	4.033	2.256
			-60mut	1.847	2.461	3.193	2.50
			-47+92	1.324	0.492	0.741	0.852
2	24/11/00	20 ul	-865+92	5.824	3.778	1.941	4.941
			-60mut	3.601	2.882	5.069	3.851
			-47+92	0.625	0.548	0.477	0.55
3	24/11/00	20 ul	-865+92	5.224	5.384	0.927	3.845
			-60mut	3.733	2.463	6.527	4.2
			-47+92	-	-	-	-
4	25/2/01	2.5×10^6	-865+92	3.214	0.345	0.336	1.298
			-60mut	14.46	8.006	2.325	8.297
			-47+92	0.248	0.767	-	0.508

Table A1.4: Actual raw luciferase values – comparison of promoter fragments –865+92, -60mut, -47+92 in the absence NGF Adult DRG cultures.

When many of the experiments were performed, the titre of the AAV samples used had not yet been established. Subsequently AAV preparations were titred and it was therefore necessary to normalise this data to the amount of virus used for each sample. Figures were normalised as follows:

Date of AAV prep	Promoter	Titre	To normalise results
4/8/00	-865+447	1.3 x 10^7/ml	× 3
	-865+92	4.0 x 10^7/ml	× 1
	-431+92a	2.8 x 10^8/ml	÷ 7
	-431+92b	3.2 x 10^8/ml	÷ 8
	-47+92	4.0 x 10^7/ml	× 1
24/5/00	-865+447	1.0 x 10^7/ml	× 3
	-865+92	2.5 x10^7/ml	÷ 1.2
	-431+92	1.8 x10^8/ml	÷ 6
	-47+92	3.0 x10^7/ml	× 1
24/11/00	-865+447	1.3 x 10^7/ml	× 3
	-865+92	4.0 x 10^7/ml	× 1
	-671+92	4.0 x 10^8/ml	÷ 10
	-431+92	4.5 x 10^8/ml	÷ 11.25
	-345+92	2.5 x 10^8/ml	÷ 12.5
	-47+92	4.0 x 10^7/ml	× 1
22/9/00	-865+447	2.0 x 10^7/ml	× 2
24/11/00	-865+92	4.0 x 10^7/ml	× 1
	-60mut	6.0 x 10^6/ml	× 6.7
	-47+92	4.0 x 10^7/ml	× 1

Table A1.5: Titres of each AAV sample and the resulting calculations used to normalise the raw luciferase values obtained.

Expt	AAV prep	Amt (infect-ious particles or µl)	Promoter	Luciferase values (relative light units)				
				1	2	3	4	Mean
1	24/11/00	20µl	-865+92	2.272	1.990	2.899	-	2.387
			-671+92	0.771	0.356	0.352	-	0.493
			-431+92	0.253	0.491	0.460	-	0.401
2	24/11/00	20µl	-431+92	0.093	0.020	0.031	-	0.048
			-345+92	0.064	0.039	0.055	-	0.053
			-47+92	N/d	N/d	0.038	-	0.013
3	24/11/00	5x10^5 particles	-865+447	2.123	0.999	0.084	-	1.069
			-865+92	1.259	0.208	0.128	-	0.532
			-671+92	0.065	N/d	N/d	-	0.022
4	4/8/00	8µl	-865+92	45.39	29.63	50.01	-	41.68
			-431+92a	11.66	9.963	16.06	-	12.56
			-47+92	1.471	1.706	-	-	1.59
5	4/8/00	8µl	-865+92	13.34	14.52	28.12	-	18.66
			431+92b	2.884	5.095	6.474	-	4.818
			-47+92	0.477	0.376	0.38	-	0.411
6	25/4/00	12µl	-865+447	78.75	9.540	9.096	-	32.46
			-865+92	4.232	4.215	2.973	-	3.807
			-47+92	0.296	0.178	0.373	-	0.28
7	25/4/00	12µl	-865+447	77.73	12.63	0.111	-	30.15
			-865+92	50.94	6.013	1.057	-	19.34
			-47+92	1.032	0.996	0.677	-	0.9
8	25/4/00	12µl	-865+447	18.51	19.60	39.96	-	26.02
			-865+92	9.496	8.403	14.02	-	10.64
			-47+92	1.437	0.902	0.182	-	0.84

9	25/4/00	15 µl	-865+92	3.270	5.358	0.451	-	3.025
			-671+92	0.547	0.135	0.601	-	0.427
			-47+92	0.651	0.124	0.308	-	0.361
10		15µl	-865+447	68.70	42.66	65.01	-	58.83
	4/8/00		-865+92	30.55	50.84	26.16	-	35.83
			-47+92	1.203	1.656	1.293	-	1.384
11	4/8/00	15µl	-865+447	211.9	77.85	138.0	-	142.6
			-865+92	62.94	101.7	84.97	-	83.2
			-47+92	1.774	0.945	1.351	-	1.36
12	25/4/00	15µl	-865+92	0.286	0.224	0.030	-	0.180
			-671+92	0.068	0.040	0.016	-	0.041
			-	-	-	-	-	-
13	24/11/00	$7x10^6$	-671+92	0.728	1.771	0.078	-	0.859
		particles	-431+92	0.33	0.459	0.936	-	0.575
	26/2/01		-47+92	0.42	0.131		-	0.276
14	24/11/00	$7x10^6$	-671+92	1.727	1.358	0.478	-	1.188
		particles	-431+92	2.564	0.948	-	-	1.756
	26/2/01		-47+92	0.204	0.153	0.04	-	0.132
15	24/11/00	$7x10^6$	-671+92	0.334	0.32	-	-	0.327
		particles	-431+92	0.895	0.275	-	-	0.585
	26/2/01		-47+92	0.107	0.056	0.032	-	0.065
16	24/11/00	15µl	-865+447	0.564	0.849	1.491	0.501	0.852
			-671+92	0.003	0.009	0.004	0.001	0.004
17	22/9/00	15µl	-865+447	0.194	0.110	0.522	0.278	0.276
	24/11/00		-431+92	0.006	0.001	0.004	0.006	0.004
18	22/9/00	15µl	-865+447	0.058	0.010	0.060	0.118	0.061
	24/11/00		-431+92	0.006	0.012	0.004	0.008	0.007

Table A1.6: Normalised raw data from **adult** DRG cultures: Actual luciferase values were normalised to the appropriate titre of AAV preparation where neccessary.

Expt	**AAV prep**	**Amt (µl)**	**Promoter**	**Luciferas values (relative light units)**				
				1	2	3	4	Mean
1	4/8/00	12	-865+447	319.2	379.8	396.0	-	365.0
			-865+92	208.9	393.9	226	-	276.0
			-431+92a	9.237	11.98	8.330	6.859	9.100
			-47+91	16.9	16.32	-	-	16.16
2	4/8/00	12	-865+447	238.2	219.0	183.2	-	213.5
			-865+92	136.3	117.9	75.63	-	109.94
			-431+92a	5.831	5.693	-	-	5.763
			-47+92	11.99	15.44	11.29	-	11.57
3	24/11/00	20	-865+447	33.97	67.98	35.29	-	59.09
			-865+92	59.3	68.5	-	-	63.9
			-671+92	7.522	2.620	-	-	5.007
			-431+92	4.945	8.293	0.578	-	4.605
			-345+92	4.092	5.795	2.248	-	4.045
4	24/11/00	20	-865+447	267.9	69.66	150.8	-	162.8
			-865+92	149.7	142	159	-	150.23
			-671+92	11.21	9.726	6.588	-	9.175
			-431+92	0.959	1.672	19.1	-	7.244
			-345+92	21.99	15.89	1.633	-	13.17
			-47+92	3.606	51.1	28.84	-	27.85

5	25/4/00	20	-865+447	5034	7320	6280	12030	7666
			-865+92	27.92	20.23	25.44	15.54	22.28
			-431+92	0.55	0.629	0.465	0.409	0.513
			-47+92	0.884	0.691	0.906	0.796	0.819
6	25/4/00	20	-865+447	1215	990	819	972	999
			-865+92	12.5	27.52	10.71	7.86	14.65
			-431+92	0.521	0.346	0.782	0.557	0.551
			-47+92	1.156	1.1014	1.549	2.205	1.481

Table A1.7: Normalised raw data from **neonate** DRG cultures: Actual luciferase values were normalised to the appropriate titre of AAV preparation where neccessary.

Expt	**AAV prep**	**Amt (infect-ious particles or μl)**	**Promoter**	**Luciferase values**			
				1	2	3	Mean
1	24/11/00	20ul	-865+92	0.98	0.648	1.228	0.952
			-60mut	0.342	1.139	0.750	0.744
			-47+92	0.124	0.178	0.158	0.152
2	24/11/00	20ul	-865+92	0.082	0.723	-	0.403
			-60mut	0.221	0.476	-	0.348
			-47+92	0.038	0.043	0.024	0.035
3	24/11/00	20ul	-865+92	10.22	16.47	4.93	10.54
			-60mut	21.84	20.37	17.76	20.03
			-47+92	2.934	1.04	1.57	1.85
4	25/2/01	2.5×10^6	-865+92	2.012	5.770	0.915	2.899
			-60mut	19.19	3.716	1.546	2.394
			-47+92	0.620	0.842	0.626	0.631

Table A1.8: Normalised luciferase values – comparison of promoter fragments –865+92, -60mut, -47+92 in the presence NGF Adult DRG cultures.

Expt	**AAV prep**	**Amt**	**Promoter**	**Luciferase values**			
				1	2	3	Mean
1	24/11/00	20 ul	-865+92	1.178	1.558	4.033	2.256
			-60mut	12.38	16.49	21.39	16.75
			-47+92	1.324	0.492	0.741	0.852
2	24/11/00	20 ul	-865+92	5.824	3.778	1.941	4.941
			-60mut	24.13	19.31	33.96	25.80
			-47+92	0.625	0.548	0.477	0.55
3	24/11/00	20 ul	-865+92	5.224	5.384	0.927	3.845
			-60mut	25.01	16.50	43.73	28.14
			-47+92	-	-	-	-
4	25/2/01	2.5×10^6	-865+92	3.214	0.345	0.336	1.298
			-60mut	14.46	8.006	2.325	8.297
			-47+92	0.248	0.767	-	0.508

Table A1.9: Normalised luciferase values – comparison of promoter fragments –865+92, -60mut, -47+92 in the absence NGF Adult DRG cultures.

Expt	AAV prep	Amt	Promoter	NGF	Luciferase values				
					1	2	3	4	Mean
1	25/2/01	2.5 x10^6	-865+92	+	0.268	0.483	-	-	0.376
				-	0.126	0.183	-	-	0.155
			-60	+	0.341	0.355	-	-	0.348
				-	1.001	1.538	-	-	1.270
2	25/2/01	2.5 x10^6	-60	+	0.27	0.566	-	-	0.418
				-	0.718	0.718	-	-	0.718
			-47+92	+	0.09	0.057	-	-	0.074
				-	0.195	0.00	-	-	0.098
3	24/11/00	20 µl	-865+92	+	0.07	0.052	-	-	0.061
				-	0.032	0.021	-	-	0.027
			-60	+	0.052	0.042	-	-	0.047
				-	0.084	0.093	-	-	0.089
4	24/11/00	20 µl	-865+92	+	43.81	49.56	-	-	46.69
				-	4.603	7.887	-	-	6.245
			-60	+	43.69	41.93	-	-	42.81
				-	21.04	34.93	-	-	27.99
5	24/11/00	20 µl	-60	+	3.528	5.338	5.206	11.08	6.288
				-	10.56	11.91	21.37	27.55	17.85

Table A1.10: Effect of NGF on AAV-60mut-LUC.

* AAV preparations generated using the two-plasmid/adenovirus method. All other preparations generated using the three plasmid protocol.

Appendix 2: Effect of growth factors on the PPT-A promoter- raw luciferase values

The effect of growth factors on the PPT-A promoter (-865+92) was examined at least three times in triplicate or quadruplicate. In addition the effect of growth factors on the CMV promoter were studied. Tables show the raw luciferase values obtained for each experiment together with mean values. The rAAV preparation and the amount of virus used for each experiment are also shown.

Expt	AAV prep	Amt. (infectious particles or µl)	NGF	Luciferase values (relative light activity)			
				1	2	3	Mean
1	25/2/01	$2.5x10^6$	-	0.003	0.002	0.040	0.015
			+	0.086	0.02	0.092	0.066
2	25/2/01	$2.5x10^6$	-	4.603	7.887	-	6.245
			+	43.81	49.54	-	46.68
3	June 1999*	10µl	-	3.5	3.74	25.56	10.93
			+	17.9	67.56	67.77	51.077
4	June 1999*	10µl	-	0.121	0.105	0.17	0.199
			+	16.83	14.76	3.08	11.56
5	25/2/01	$2.5x10^6$	-	2.388	2.914	3.622	2.97
			+	5.104	5.446	11.84	7.483

Table A2.1: Effect of NGF on the PPT-A promoter

Expt	AAV prep	Amount (µl)	GDNF	Luciferase values (relative light units)				
				1	2	3	4	Mean
1	June 1999*	5	-	66	17.14	10	-	33
			+	269.2	85.7	77.96	-	144.29
2	11/3/00	10	-	31.56	17.14	1.771	-	16.82
			+	75.38	53.56	-	-	64.47
3	10/4/00	10	-	5.527	2.407	0.782	0.718	2.61
			+	10.32	4.854	4.431	6.527	6.533
4	25/4/00	10	-	1.047	0.649	0.36	-	0.685
			+	2.861	1.172	1.038	-	2.357

Table A2.2: Effect of GDNF on the PPT-A promoter

Expt	AAV prep	Amount (µl)	-GF/ +LIF/ +LIF +NGF	Luciferase actvity (relative light units)			
				1	2	3	Mean
1	11/3/00	5	- GF	36.51	33.4	32.41	34.107
			+LIF	23.43	14.49	13.47	17.13
			+LIF +NGF	106.3	41.05	9	52.117
2	11/3/00	5	-GF	4.642	2.032	1.164	2.553
			+LIF	-	-	-	-
			+LIF +NGF	11.67	8.509	8.387	9.522
3	25/4/00	5	-GF	12.42	2.726	0.972	5.594
			+LIF	3.627	0.427	0.175	1.41
			+LIF +NGF	1.796	1.309	0.896	1.027
4	10/4/00	10	-GF	11.54	2.1	1.053	4.898
			+ LIF	2.584	1.174	0.872	1.543
			+LIF +NGF	2.338	2.158	1.147	1.881
5	10/4/00	15	-GF	0.231	0.229	0.038	0.166
			+LIF	0.113	0.089	0.056	0.086
			+LIF +NGF	0.223	0.175	0.056	0.151
6	10/4/00	15	-GF	0.613	0.59	0.401	0.535
			+LIF	0.409	0.233	0.144	0.262
			+LIF +NGF	2.44	2.038	0.159	1.66

Table A2.3: Effect of LIF and NGF on the PPT-A promoter

Expt	AAV prep	Amout (µl)	BDNF	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	22/9/00	12	-	0.512	0.463	0.365	0.243	0.396
			+	2.133	1.209	0.212	0.16	0.929
2	22/9/00	12	-	8.652	1.903	1.827	1.725	3.527
			+	4.453	3.643	2.621	1.696	3.103
3	22/9/00	15	-	0.965	0.868	0.51	-	0.781
			+	1.448	1.326	1.006	-	1.260
4	25/4/00	15	-	0.053	0.042	0.052	0.042	0.047
			+	0.173	0.083	0.097	0.187	0.135

Table A2.4: Effect of BDNF on the PPT-A promoter

Expt	AAV prep	Amt. (infect-ious particles or µl)	IL-6	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	11/3/00	5 µl	-	101.2	81.04	20.87	13.96	54.268
			+	111.2	99.3	97.51	45.56	88.393
2	11/3/00	5 µl	-	6.932	2.568	2.162	0.124	4.467
			+	9.269	5.611	2.797	2.528	5.051
3	25/2/01	$2.5x10^6$	-	3	2.029	1.666	1.389	2.021
			+	28.56	12.05	4.3	3.495	12.10

Table A2.5: Effect of IL-6 on the PPT-A promoter

Expt	AAV prep	Amt. (µl)	NGF	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	24/11/00	15	-	0.217	0.343	0.318	0.299	0.294
			+	0.272	0.410	0.169	0.253	0.276
2	24/11/00	15	-	0.342	0.524	0.374	0.335	0.394
			+	0.422	0.649	0.667	0.501	0.560

Table A2.6: Effect of NGF on the CMV promoter

Expt	AAV prep	Amt. (µl)	GDNF	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	24/11/00	15	-	0.645	0.434	0.770	2.005	0.966
			+	0.917	1.083	0.748	0.706	0.864
2	24/11/00	15	-	0.288	0.211	0.621	0.123	0.311
			+	0.399	0.463	0.744	0.749	0.589

Table A2.7 Effect of GDNF on the CMV promoter

Expt	AAV prep	Amt. (µl)	LIF	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	24/11/00	15	-	0.108	0.124	0.114	0.138	0.121
			+	0.213	0.238	0.121	0.087	0.165
2	24/11/00	15	-	0.154	0.334	0.208	0.121	0.204
			+	0.208	0.267	0.190	0.229	0.224

Table A2.8 Effect of LIF on the CMV promoter

Expt	AAV prep	Amt. (µl)	BDNF	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	4/8/00	15	-	1.023	1.460	2.089	1.004	1.394
			+	0.977	0.663	1.097	0.646	0.846
2	4/8/00	15	-	1.548	2.056	1.802	0.698	1.526
			+	1.374	3.352	1.042	-	1.923

Table A2.9 Effect of BDNF on the CMV promoter

Expt	AAV prep	Amt. (µl)	IL-6	Luciferase values (relative light activity)				
				1	2	3	4	Mean
1	24/11/00	15	-	1.265	1.072	1.010	0.422	0.942
			+	0.464	0.479	0.745	1.462	0.788
2	24/11/00	15	-	1.351	1.660	1.812	1.224	1.512
			+	2.359	3.223	2.403	1.707	2.426

Table A2.10 Effect of IL-6 on the CMV promoter

Expt	AAV prep	Amt	NGF+	Luciferase values (relative light activity)						
				1	2	3	4	5	6	Mean
1	11/3/00	5 µl	-	1559	1567	1133	1567	1758	1623	1535
			LIF	2571	1848	2688	2050	1694	2206	2171
2	11/3/00	5 µl	-	2.272	3.647	0.603	0.17	-	-	1.673
			GDNF	53.66	10.21	4.992	5.224.	-	-	18.52
			LIF	2.846	5.181	7.616	16.59	-	-	8.058
3	11/3/00	5 µl	-	117.9	75.63	236.3	-	-	-	109.9
			LIF	49.39	55.66	99.66	-	-	-	68.24
4	11/9/99*	10 µl	-	1.482	1.246	1.08	-	-	-	1.269
			BDNF	0.437	0.545	0.509	-	-	-	0.497
			GDNF	0.344	0.371	0.341	-	-	-	0.352
			BDNF+ GDNF	2.195	1.589	1.552	-	-	-	1.779
5	11/9/99*	10 µl	-	0.788	0.906	0.882	-	-	-	0.859
			BDNF	0.501	0.492	0.519	-	-	-	0.504
			NT-3	1.00	0.926	0.680	-	-	-	0.845
			BDNF+ NT-3	0.438	0.486	0.609	-	-	-	0.511
6	11/9/99*	10 µl	-	0.863	0.865	0.73	-	-	-	0.819
			BDNF	0.418	0.448	0.518	-	-	-	0.461
			GDNF	1.187	1.132	0.514	-	-	-	0.944
			BDNF+ GDNF	0.429	0.462	0.159	-	-	-	0.350
7	11/9/99*	10 µl	-	0.413	0.879	1.08	-	-	-	0.791
			CNTF	1.214	0.682	0.868	-	-	-	0.921
			GDNF	0.857	0.534	0.868	-	-	-	0.753
			CNTF+ GDNF	0.88	0.513	0.545	-	-	-	0.646
8	11/9/99*	10 µl	-	0.716	0.359	0.397	-	-	-	0.491
			NT-3	0.25	0.865	0.37	-	-	-	0.495

Table A2.11: Neonate GF results

* AAV preparations generated using the two-plasmid/adenovirus method. All other preparations generated using the three plasmid protocol.

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